Universidade de Aveiro Departamento de Química
Ano 2014

Ana Cláudia Marques Lopes Soares da Costa

The effect of dietary fatty acid on breast cancer lipidome.

Efeito dos ácidos gordos da dieta no lipidoma do cancro da mama

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessarios à obtenção do grau Mestre em Bioquimica, realizada sob a orientação cientifica da Doutora Maria do Rosario Gonçalves Reis Marques Domingues, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Luísa Alejandra Helguero Sheperd, Investigadora Auxiliar do Departamento de Química da Universidade de Aveiro.

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Palavras-chave Fosfolípidos, ácidos gordos da dieta, cancro da mama, espectrometria de massa, lipidómica

Resumo

O cancro da mama é uma doença heterogênea complexa e uma das principais causas de morte entre as mulheres. Muitos estudos têm tentado determinar se ácidos gordos (FA) saturados, monoinsaturados e polinsaturados têm efeitos diferentes sobre o desenvolvimento de cancro da mama. No entanto o papel dos diferentes FAs da dieta no lipidoma de células de cancro da mama não é completamente compreendido. O objetivo deste trabalho foi avaliar se o lipidoma da linha celular T-47D de cancro da mama se altera com diferentes FAs da dieta e correlacionar essas alterações com efeitos na proliferação e apoptose celulares. As alterações lipídicas induzidas pela presença de 1 ug / mL de ácido oleico (OA1) ou 1 ou 2 ug / ml de ácido linoleico (LA1 ou LA2, respetivamente) de ácido *in vitro* foram estudadas através de uma análise lipidómica com base em técnicas tais como TLC, para a separação e quantificação de classes de fosfolípidos (PLs) e GC-MS, para separação e quantificação dos FAs. Os triglicerídeos (TGs) e colesterol (CHL) totais foram quantificados utilizando métodos colorimétricos. Além disso, a viabilidade celular e a apoptose também foram avaliadas. A análise de classes de PL permitiu observar alterações significativas nas classes de fosfatidiletanolamina (PE), fosfatidilserina (PS) nas células suplementadas com LA1 e LA2, respetivamente, e lisofosfatidilcolina (LPC) nas células suplementadas com OA1. Observou-se ainda um aumento do FA 18:2 no tratamento com LA. Além disso, os TGs mostraram uma diminuição no tratamento com LA2. Os ensaios de viabilidade e apoptose mostraram que LA1 e LA2 estimulam o crescimento celular e que OA2 estimula a apoptose. Este trabalho contribui para uma melhor compreensão da influência dos FAs da dieta no lipidoma de células T-47D e mostra que as alterações no lipidoma celular estão associadas a um aumento do metabolismo e crescimento celular nas células suplementadas com LA e a um aumento da apoptose em células suplementadas com OA. Essas descobertas podem levar a novas perspetivas na identificação de estratégias específicas que podem ser úteis para compreender o processo envolvido na progressão do cancro da mama. No entanto, mais estudos são necessários para concluir sobre como os diferentes lípidos podem promover estes efeitos.

Keywords Phospholipids, dietary fatty acids, breast cancer, mass spectrometry, lipidomics

Abstract

Breast cancer is a complex heterogeneous disease and one of the leading causes of death among women. Many studies have tried to determine if saturated, monounsaturated and polyunsaturated fatty acids (FA) have different effects on the development of breast cancer. However, the role of different dietary FAs in lipidome of breast cancer cells is not completely understood. The aim of this work was to establish if lipidome of breast cancer T47-D cell line differs with different dietary FAs and correlate this findings to effects in proliferation and apoptosis. The lipid changes in lipidome induced by the presence of 1 µg/mL oleic acid (OA1) or 1 or 2 µg/mL linoleic (LA1 or LA2, respectively) acid *in vitro* were study through a lipidomic analysis, based on techniques such as TLC and GC-MS. TLC allowed the separation and quantification of Phospholipid (PL) classes and GC –MS allowed the separation and quantification of FAs. TGs and CHL were quantified. Also, cell viability and apoptosis were evaluated. PL class analysis showed significant alterations in phosphatidylethanolamine (PE), phosphatidylserine (PS) in the medium with LA1 or LA2, respectively and lysophosphatidylcholine (LPC) in the medium with OA1. GC-MS showed an increase of 18:2 in the treatment with LA. Further TG showed a decrease on LA2 treatment and viability and apoptosis assays showed that LA1 and LA2 stimulate cell growth and OA2 stimulated apoptosis, respectively. This work contributes to a better understanding of the influence of dietary FAs on the lipidome of T-47D cells and shows that alterations in cellular lipidome are associated to changes in cell number and apoptosis. These findings may lead to new perspectives in the identification of specific targets that may be helpful to understand the process behind breast cancer growth. However, more studies are necessary to conclude about how different lipid molecules promote these effects.

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Abbreviations:

AA – arachidonic acid
ACBP – acyl-CoA binding protein
ALA – alfa -linolenic acid
ATX – autotaxin
BSA – Albumin bovine serum
C1P – ceramide-1-phosphate
CAM – cell adhesion molecules
CDP – cytidine diphosphate
CDP-DIP – CDP- DAG inositol phosphatidyltransferase
CHKA – choline kinase alpha
CHL – cholesterol
CL – cardiolipin
COX – cyclooxygenase
CYP 450 – cytochrome P450
DAG – diacylglycerol
DHA – docoesopentaenoic acid
DMSO – dimethylsulfoxide
ECM – extracellularmatrix
EDTA – Ethylenediaminetetraacetic acid
EFA – essential fatty acids
EGF – epithelial growth factor
EK – ethanolamine kinase.
EPA – eicosapentaenoic acid
EPT – CTP:phosphoethanolaminecytidylyltransferase
ER – estrogen receptor
ET – CTP:phosphoethanolaminecytidylyltransferase
FA – fatty acids
FABP - fatty acid-binding protein
FBS- fetal bovine serum
FFA - free fatty acids
FITC - fluorescein isothiocyanate
GC – gas chromatography

GP – glycerophospholipids

GPCR – G-protein-coupled receptor

HER2 – human epidermal growth factor receptor 2

HER2-OE – HER2-overexpressing

HPETE – hydroxyperoxyeicosatetraenoic acid

HPLC – high performance liquid chromatography

HUFA – highly unsaturated fat acid

IP3 – inositol (1,4,5)-trisphosphate

LA – linoleic acid

LC – liquid chromatography

LOX – lipoxygenase

LPA – lysophosphatidic acid

LPC – lysophosphatidylcholine

LPCAT1 – lysophosphatidylcholine acyltrasferase1,

LPE – lysophosphatidyletanolamine

LPG – lysophosphatidylglycerol

LPI – lysophosphatidylinositol

LPS – lysophosphatidylserine

MAP – mitogen-activated protein (MAP)

MMP – matrix metalloproteinase

MS – mass spectrometry

MTBE - methyl -tert-Butyl Methyl

MUFA – monounstaturated fatty acid

OA1 - 1ug/mL Oleic acid

OA2 - 2ug/mL Oleic acid

PA – phosphatidic acid

PAF – platelet activating factor

PBS - phosphate buffered saline

PC – phosphatidylcholine

PE – phosphatidylethanolamine

PEST - Protein Tyrosine Phosphatase

PG – phosphatidylglycerol

PI – phosphatidylinositol

PI3K – phosphatidylinositol 3 kinase

PI-5-K – phosphatidylinositol 5-kinase

PIP – phosphatidylinositol phosphate

PIP2 – phosphatidylinositol 4,5-bisphosphate

PIP3 – phosphatidylinositol 3,4,5-trisphosphate

PL – phospholipid

PLA2 – phospholipase A2

PLC – phospholipase C

PLD – phospholipase D

PR – progesterone receptor

PS – phosphatidylserine

PSD – phosphatidylserinedescarboxilase

PSS1 – phosphatidilserine synthase 1

PSS2 – phosphatidilserine synthase 2

PTEN – Phosphatase and tensin homologue

PUFA – polyunsaturated fatty acid

S – sphyngomielinase

S1P – sphingosine – 1 – phosphate

SFA – saturated fatty acid

SM – sphingomyelin

SMS – sphyngomilelin synthase

SPC – sphingosylphosphorylcholine

TDLU – terminal duct lobular units

TFA – trans fatty acids

TG – triglyceride

TLC – thin layer chromatography

I. Introduction

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1. The mammary gland

The mammary gland is a very specialized exocrine gland, located in the breast, with the specific function of synthesizing, secreting, and delivering milk to the newborn (1). It is composed by multiple types of cells that together form complex interaction networks required for the proper development of the organ (2).

The completely developed breast has 15 to 20 sections (the lobes), and each lobe is made of many smaller sections (lobules) that are composed by alveoli, where milk is produced and secreted through a system of ducts that transport the milk from the alveoli to the nipple. Ducts terminate in terminal duct lobular units (TDLU), the anatomical and functional units of the breast (Figure 1). Two cellular compartments contribute to the gland: the epithelium and the surrounding stroma (e.g. endothelial cells, fibroblasts, myofibroblasts, and leukocytes), which are derived from ectoderm and mesoderm, respectively (3). The epithelial cells that compose the gland are arranged in two layers, an inner luminal epithelial layer and an outer myoepithelial layer (4). The luminal cells are responsible for producing milk during lactation, while the myoepithelial cells in contact with the basement membrane assist in milk ejection during lactation and provide structural support to the lobules (5).

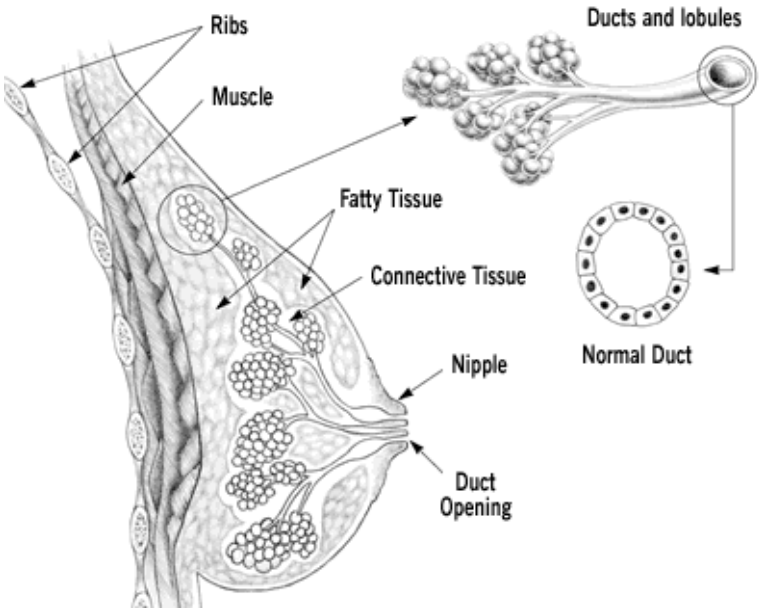

Figure 1. Schematic representation of the anatomy of the breast. Adapted from www.my-breast-cancer-guide.com/what-is-breast-cancer.html

The mammary gland, unlike most other organs, is not fully formed at the time of birth, reaching its full differentiation only during a pregnancy/lactation cycle, via hormonal influences. These influences promote drastic modifications in the anatomy of the gland, resulting in the remodeling of the gland into a milk-secretory organ (1). There are key stages in mammary development that exhibit considerable structural and functional variations throughout lifetime, particularly during puberty, normal menstrual cycle, pregnancy and lactation, and at menopause (6,7). Until puberty only a rudimentary ductal system exists. It is at the beginning of puberty, that more dynamic and pronounced changes in the breast occur with the establishment of menstrual cycle regulated by the ovarian and pituitary hormones (8). In the first half of the menstrual cycle the lobules are relatively quiescent. After ovulation, under the influence of female sex hormones, cell proliferation increases and so do the number of alveoli in each lobule (estrogen leads to proliferation of ductal cells and progesterone leads to increased proliferation and differentiation of alveolar cells). Upon menstruation, the decrease in the levels of estrogen and progesterone induces regression of the lobules and the disappearance of the stromal edema. Only with the onset of pregnancy the breast becomes completely mature and functional. Lobules increase progressively in number and size. Immediately after the baby is born, the luminal cells of the lobules produce colostrum, which then changes to milk. Cessation of lactation after weaning leads to extensive apoptosis of epithelial cells and tissue remodeling and the total breast size decreases considerably but full regression does not occur (9). Finally, in involution, being triggered by a declining of ovarian function and by a decrease in circulatory levels of estrogen and progesterone, the lobules and their specialized stroma continue to regress and atrophy and the adipose tissue increases (1).

A balance between proliferation, differentiation, and death in the stem-cell population and throughout the cells of the mammary gland is critical for normal development. Alterations of normal proprieties of cells derived from a series of molecular events can lead to breast cancer.

2. Breast cancer

Breast cancer is a complex heterogeneous disease and one of the leading causes of death among women in the world, despite the significant advances in diagnosis and treatment. The manifold unresolved problems that remain are related to prevention, diagnosis, tumor progression and recurrence, treatment and therapeutic resistance. It is

very difficult to overcome these problems, because breast cancer is not a single disease but a highly heterogeneous collection of different diseases which affect the same anatomical organ and is originated in the same anatomical structure, TDLU (10,11). The causes of breast cancer are not yet fully known, although a number of risk factors that modulate the development of breast cancer have been identified. Among the risk factors, the genetic causes associated to familial forms of breast cancer account for only 5-10% of breast cancer. Therefore, most of the risk factors leading to breast cancer development are either environmental or related to life-style (12).

Breast cancer can be classified taking into consideration different aspects such as, histopathology, grade of the tumor, stage of the tumor and, protein and gene expression. Histopathologic evaluation of a breast cancer by biopsy studies is necessary to provide the diagnosis of the tumor. Most breast cancers are derived from the epithelium lining the ducts or lobules, and these cancers are classified as ductal or lobular carcinoma, respectively. The most common type of breast cancer is ductal cancer. Cancers also are classified as non-invasive (*in situ*), that refers to cancer that confine itself to the tissue compartment where it initially developed and as invasive (infiltrating), that has a tendency to spread (metastasize) to other areas of the breast and/or other regions of the body (13,14); the grade of the tumor is a system that is based on the loss of differentiation capacity and it's divided into three distinct grades: Grade I - well differentiated (is low grade and resembles normal tissue), Grade II - moderately differentiated (intermediate grade) and Grade III - poorly differentiated (is composed of disorganized cells and, therefore, does not look like normal tissue). A higher histological grade corresponds to a worse prognosis (15); the stage of the tumor that is based on tumor size is a prognostic factor with a high impact. The larger the size of the tumor, the greater the probability of occurrence of clones with the ability to metastasis and consequently the worse is the prognosis; the molecular classification of breast cancer by gene-expression profiling established major breast intrinsic subtypes: The luminal A and B (expressing estrogen receptors; ER), the HER2-overexpressing (HER2-OE), claudin-low and basal-like (16)(14). Such classification is useful because the patients, in general, are treated according to the type of receptors that cells express (i.e. ER antagonists, HER2 inactivating antibodies). Luminal type can have expression of estrogen receptor (ER), progesterone receptor (PR) and varying degrees of human epidermal growth factor receptor 2 (HER2). Luminal A is characterized by the expression of ER and PR in cancer cells, whereas the luminal B is characterized by positivity of ER and PR together with

HER2 overexpression and/or high rates of cell proliferation (15,17). HER2-OE tumours are negative for hormonal receptors and overexpress HER2 protein (associated with HER2/neu gene amplification)(15). Basal -like tumors are the less differentiated and are characterized by the absence of ER, PR and HER2 (also called “triple-negative”) and presence of basal cytokeratins, and highly associated with specific aggressive histological features and poor patient survival (18). Claudin-low subtype, is mostly triple negative but with less cell – cell adhesion proteins and frequent infiltration of lymphocytes (16). Each of this four aspects (histopathology, grade of the tumor, stage of the tumor and, protein and gene expression), influences the prognosis and the therapies that are chosen for treatment.

As mentioned above, breast cancer is a cellular disease characterized by uncontrolled cell proliferation that can be spread to various tissues in the body through a process known as metastasis. This process consists of several steps that need to be completed successfully to form a metastatic tumor (Figure 2).

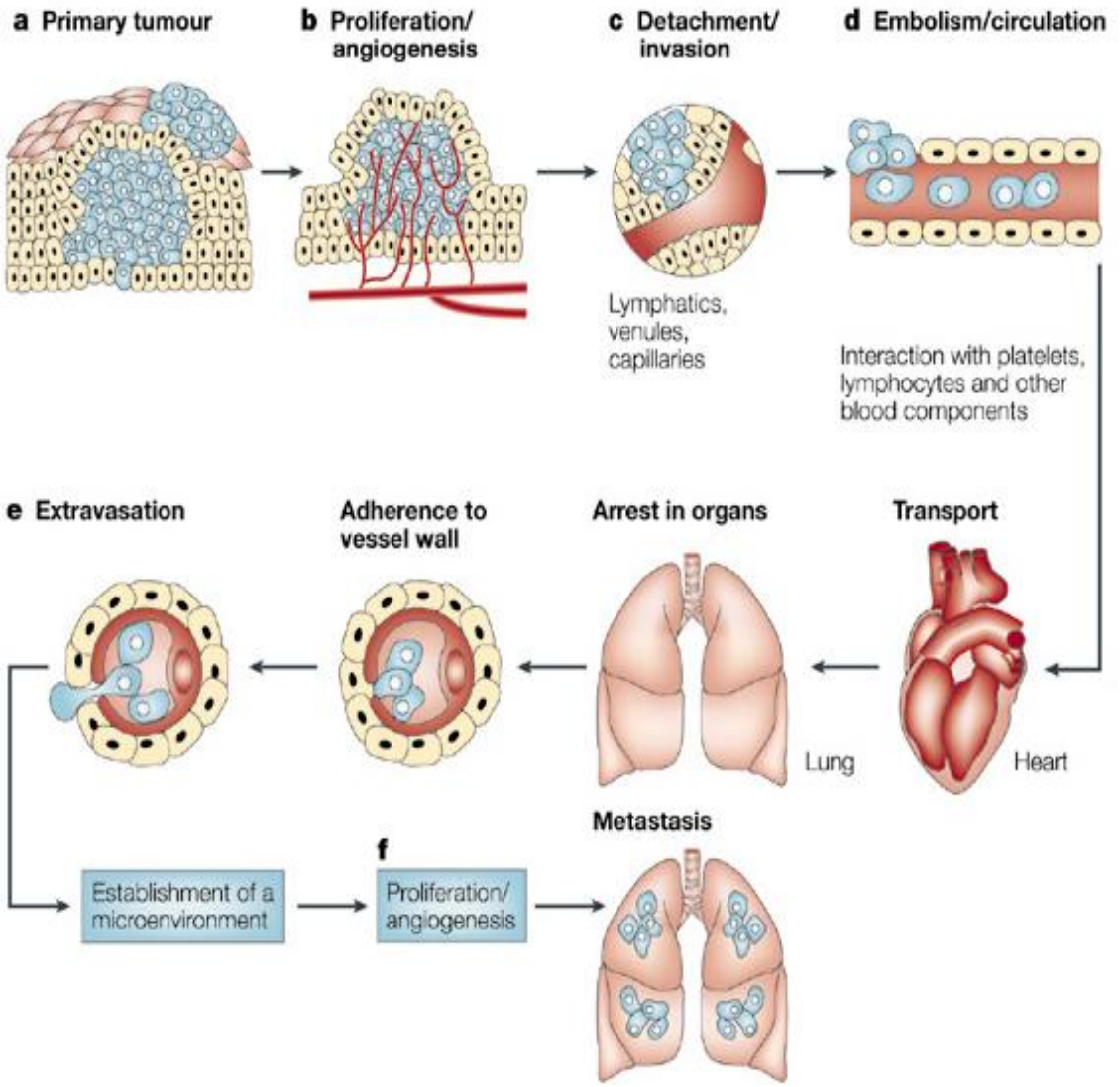

Figure 2. Tumor metastasis step-by-step process. a. cellular transformation and tumor growth; b. extensive vascularization of the tumor; c. local invasion of the host stroma by some tumor cells; d. detachment and embolization of single tumor or aggregates and the cell tumors that survive to the immune system become trapped in the capillary beds of distant organs; e. extravasation occurs; f. proliferation within the organ completes the metastatic process. Adapted from (19).

In the *process* of invasion and *metastasis*, tumor cells leave its original location (primary tumor) to a new part of the body. As cancer cells divide, they can invade and grow directly into the surrounding tissue or structures (direct extension), but they can also spread by breaking away from a breast tumor. If the cancer cells are not detected by the immune system, they can enter into the bloodstream (blood vessels) or lymphatic system (lymph vessels). Once in the circulation, cells can be transported over long distances

reaching other parts of the body (20). At particular sites, depending on the type of cancer, cells adhere to blood vessel walls, extravasate and seed in target organs. Some cells remain dormant for a long time before they start the formation of metastatic foci (19).

It is probable that the interaction with the microenvironment contributes to the behavior of tumor cells and a major component is the extracellular matrix (ECM) (21) . The ECM consists of collagens, laminins and fibronectin and serves as structures which cells can attach and migrate. Cell – cell and cell – extracellular matrix (ECM) interactions are very important in the control of progression and migration of tumor cells and these interactions are mediated by proteins known as cell adhesion molecules (CAMs) (21,22). CAMs, a diverse system of transmembrane glycoproteins, are located on the cell surface and can be classified into five families including immunoglobulin superfamily, integrins, cadherins, selectins and CD44. These receptor proteins are composed by three domains: an intracellular domain which interacts with the cytoskeleton, a transmembrane domain and an extracellular domain that interact with other CAMs or the ECM allowing cells to communicate (22). To be functional they need to be activated, so cadherins and integrins need to form clusters. The cluster formation begins with the grouping of many CAM molecules in the plasmatic membrane, allowing the molecules to bind their ligands with higher strength. Formation of clusters and CAM activation allows them to transduce information from the ECM to the cell as well as reveal the status of the cell to the outside, allowing rapid and flexible responses to changes in the environment. Alterations on CAMs play an important role in tumor dissemination, because migration of cancer cells is critically regulated by physical adhesion of cells to each other and to their non-cellular surroundings. Loss of intercellular adhesion allows malignant cells to detach and to escape from the primary mass (22). Gaining a more motile and invasive phenotype, these cells secrete matrix metalloproteinases (MMP) which degrades the collagen of the ECM breaking through the cellular membrane that surrounds the tumor, allowing the tumor cell to migrate towards the blood or lymph vessels and eventually invade and metastasize to distal organs (23).

Changes in membrane lipid composition have been reported in breast cancer. This changes in the lipid of the cell can regulate function and availability of intrinsic membrane proteins such as CAMs, thus affecting inside out and outside in cell signaling. This issue will be talked about further in the next section.

3. Lipids

Lipids are essential components of the cell and play multiple and distinct roles in cellular functions. There is a great diversity of lipids, with very different structure and functions. The majority of the lipids of the cells form a lipid bi-layer whose characteristics are essential for the structural integrity and functionality of the cell membrane. Membrane lipids, particularly phospholipids (PLs), play key roles in protein assembly, allowing protein trafficking and anchoring to the membrane and provide a hydrophobic environment for membrane proteins function and interaction (24). The lipids of the membrane are involved in signal transduction. Besides, lipids are also energy storage molecules. For all these reasons, lipids are nowadays considered important players in membranes and in signalling, not only due to their own functions, but also for conditioning the functions of proteins in the cells (24). Thus it has become very important to deepen the knowledge concerning lipid profiles and lipid metabolism as well as how it changes with disease.

Traditionally lipids are defined based on their solubility, since they are insoluble in water and soluble in organic solvents (such as chloroform, ether, acetone, among others). They also have in common the presence of long chains of hydrocarbons. However the diversity of lipids is so large that does not allow a simple definition. In fact a recent classification reports 8 classes based on their chemical structure: fatty acids (FAs), glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, prenol lipids and sterol lipids (25). Several classes are divided into subclasses and each class embraces a molecular diversity of species due to the different combinations of distinct FAs with different length and instauration/saturation.

One of the main classes of lipid membranes are the PLs, amphipathic compounds, that are the major component of most biological membranes of all cell forming lipid bilayers (Figure 3) (26). Also cholesterol (CHL), the principal constituent of group of sterol lipids is an important constituent of membrane, being signaling regulation and membrane fluidity their main function (26).

Glycerolipids include fundamentally monoacylglycerols, diacylglicerols (DAG) and triglycerides (TG). TGs are an esters derived from glycerol and three FAs, and they are very abundant in nature, being the main constituents of animal fat. TGs have an important role in cellular energy storage (27).

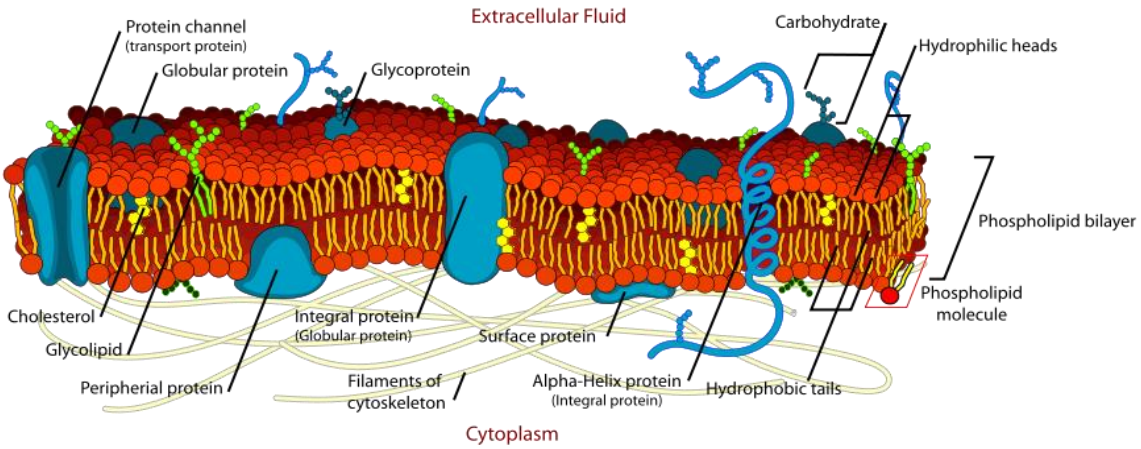

Figure 3. Illustration of a eukaryotic cell membrane. Adapted from http://einstein.sc.mahidol.ac.th/~bionanotech/7_Research_Page/ResProj_9/Physics_of_Cell_Membrane.html

3.1. Fatty acids

FAs are the basic elements of most lipids. Biological systems can have free FAs (FFAs), or they can be esterified into other lipids classes. FFAs are carboxylic acids represented by the formula R-COOH, where R is usually a chain of hydrocarbon radical, un-ramified and, in general, these FAs have an even number of carbon atoms joined by single or double bonds. FAs can be classified according to the degree of saturation, total number of carbons and whether they are synthetized by the organism by *de novo* lipogenesis or must be consumed in the diet (essential FAs) (28,29).

In the circulation, FAs are transported bound to albumin or as part of lipoproteins and they are taken up into cells mainly by protein carriers in the plasma membrane and then transported in the cell via fatty acid-binding proteins (FABP). Inside the cells, FFAs are activated (acyl-CoA) to then be transported to the mitochondria or peroxisomes or b-oxidation to obtain energy, or to the endoplasmic reticulum for esterification to different lipid classes. Acyl-CoA or certain FFAs may bind to transcription factors that regulate gene expression or may be converted to signaling molecules (eicosanoids) (29) (Figure 4).

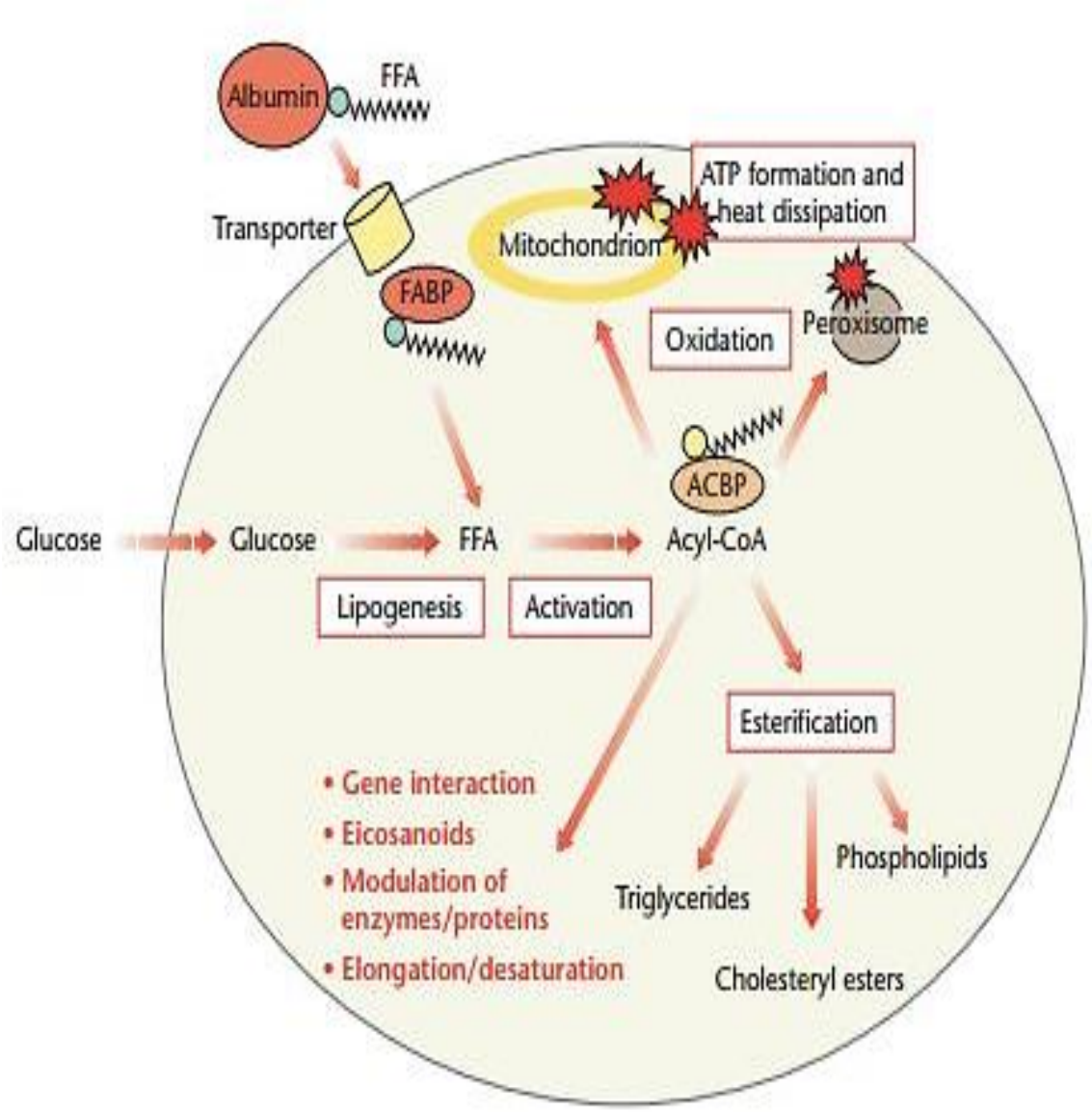

Figure 4. The metabolism of FAs. FAs can come from the diet or *de novo* lipogenesis once they are activated (acyl-CoA), they are either incorporated into membrane lipids (PL), into storage lipids (TG and CHL esters) or suffer oxidation in mitochondrion or peroxisome to form energy. Also, FAs can be important for other functions in the cell such as ligands capable of activating gene expression. Adapted from (29).

The FAs can be subdivided into 3 classes depending on the degree of saturation. They can be saturated FAs (SFA), which have no double bonds, monosaturated FAs (MUFA) that have one double bond, and polyunsaturated FAs (PUFA) that have two or more double bonds. The double bonds of naturally occurring unsaturated FAs are very often of the *cis* orientation. This means that the hydrogen atoms attached to the double

bonds are on the same side. However if the hydrogen atoms are on opposite sides, the configuration is termed *trans*(29).

The SFA can occur in many kinds, which differ mainly in the number of carbon atoms such as butyric (C4:0), caproic (C6:0), caprylic (C8:0), capric (C10:0), lauric (C12:0), myristic (C14:0), palmitic (C16:0), and stearic (C18:0) acids. C16:0 is usually considered the most abundant SFA in nature, and it is found in appreciable amounts in the lipids of animals, plants and lower organisms. C18:0 is the second most abundant SFA in nature (29). Relatively high proportions of stearic acid are subjected to enzymatic desaturation (to oleic acid), in comparison to other SFAs.

The most common MUFA is oleic acid (OA) and it is present in considerable quantities in both animal and plant sources. These FA is obtained through diet but can be also synthesized by mammals. The biosynthesis of OA involves the action of enzyme CoA 9-desaturase. This enzyme, present in plants and animals, induces a double bond in the ninth position from the hydrocarbon terminal, converting C18:0 in OA (C18:1, n-9) (29).

The PUFAs are classified in three families according to the position of the first double bond from the methyl group terminal of the molecule (n-9, n-6 and n-3) (30). Linoleic acid (LA) (18:2 n-6) and alpha-linolenic acid (ALA) (C18:3 n-3) are essential FAs (EFAs) (Figure 5). EFAs can only be obtained through the diet, due to the absence, in mammals, of their biosynthetic enzymes (28). Humans are able to convert LA and ALA to more physiologically active FAs through a series of elongation and desaturation reactions (28). LA can be converted to arachidonic acid (AA; 20:4 n-6) and ALA converted to eicosapentaenoico acid (C20:5 n-3)(EPA) and docosapentaenoic acid (C22: 6 n-3) (DHA) (Figure. 5) (29). FA of different families do not interconvert.

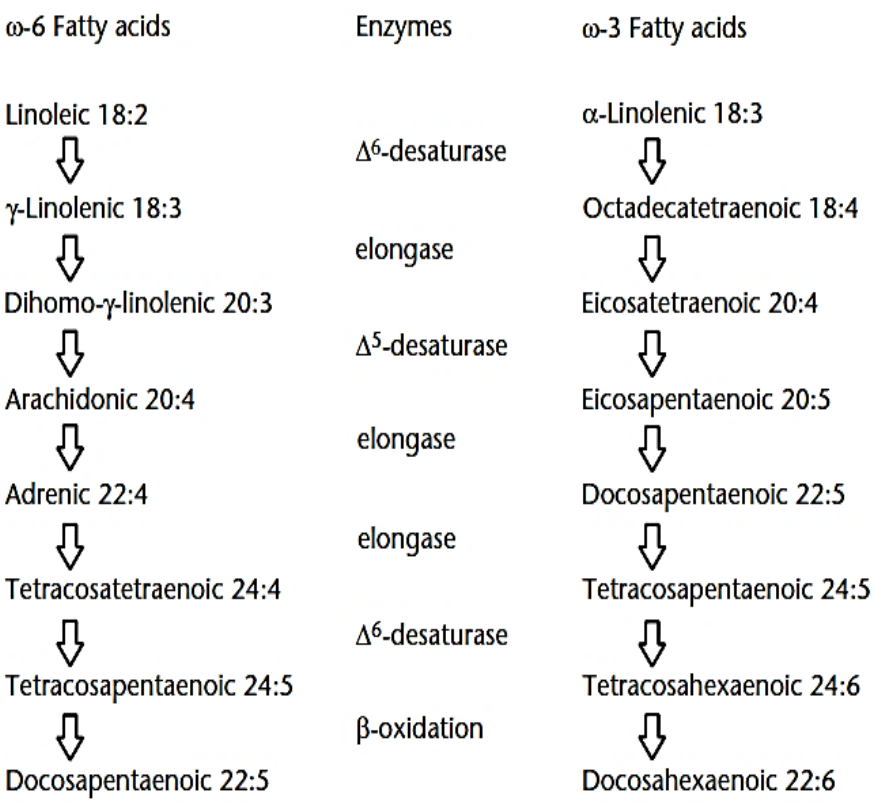

Figure 5. Biosynthetic pathways of Omega-3 and omega-6 PUFAs with the indication of the main enzymes that participate in these biosynthetic routes. Each metabolic sequence competes for the same enzyme systems, with the affinity being greater for *n*-3 FA than *n*-6 FA. Adapted from (29).

The unsaturated FAs are incorporated into the *sn-2* position of membrane PLs (that will be reported latter), with degrees of affinity to respect the order n-3> n-6> n-9 differently influencing the structure and function of different receptors, transporters, enzymes and ion channels associated with the membrane while SFAs are preferentially located at *sn-1* positions (30).

The AA, EPA and DHA are also classified as highly unsaturated fat acids (HUFAs) which perform several essential functions in the body. EPA and AA are FAs of the families n-3 and n-6, respectively. EPA and AA can be released from PLs by the action of phospholipase A2, and metabolized by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 enzymes in different eicosanoids. COX metabolism leads to formation of prostaglandins and thromboxanes, while LOX leads the formation of leukotrienes and CYP 450 hydroxyl or epoxide the double bonds to form hydroxy- and epoxy PUFAs. These are important mediators of the inflammatory process (28).

Trans FAs (TFAs) are a group of unsaturated FAs with unique shapes and properties. Humans do not synthesize *trans*-FAs, they are obtained from the diet. Although naturally occurring *trans*-fat exists (ex. Conjugated linoleic acid), the main source of this type of FAs is dietary intake of industrialized products containing partially hydrogenated oils (31). These acids are originated from the cis isomers, that are modified during the chemical process (32).TFAs of the same length and weight as the original *cis* FAs, still have the same number of carbons, hydrogens, and oxygens but they are now shaped in a more linear form. This process converts oily bodies into semiliquid or solid fats for subsequent use in food products and the result of incorporation of hydrogen is a product with more oxidative stability (33).

3.2. Phospholipids

PLs are amphipathic molecules that consist of a hydrophilic head of phosphate and a hydrophobic tail composed by FA chains, thus tending to form lipid bi-layers. In fact, they are the main constituents of all biological membranes and allow establishing the organized structure of the membrane. These lipids confer a relatively rigid structure to the membrane, which confers protection and resistance to the cells. The PLs of membrane gives support for proteins, channels and other lipids, allowing exchanges between the internal and external environment. In addition to components of the membrane, PLs are also involved in other functions specially as signaling molecules (24). The metabolites resulting from the biosynthesis and degradation of PL are extremely important to the intracellular signaling and may be involved in processes of proliferation and apoptosis.

There are two main classes of PL, one that has a glycerol backbone and other that contains sphingosine. PLs that contain glycerol backbone are called glycerophospholipids (GPs). The GPs are the major components of the membranes, and some have intrinsic biological activity acting as a second messengers. Structurally, these consist of a glycerol backbone linked by ester linkages to two FA chains in the positions sn-1 and sn-2, which may have similar or different characteristics (saturated, monounsaturated or polyunsaturated). In sn-3 position of the glycerol is esterified to a molecule of phosphate, which can bind, by phosphodiester linkage, to a polar molecule forming the PL polar head (24). Depending on the molecule which binds to the phosphate, different classes of GP

may occur: phosphatidic acid (PA), phosphatidylcholine or lecitin (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), Phosphatidylinositol (PI) (Figure 6) (24,34) .

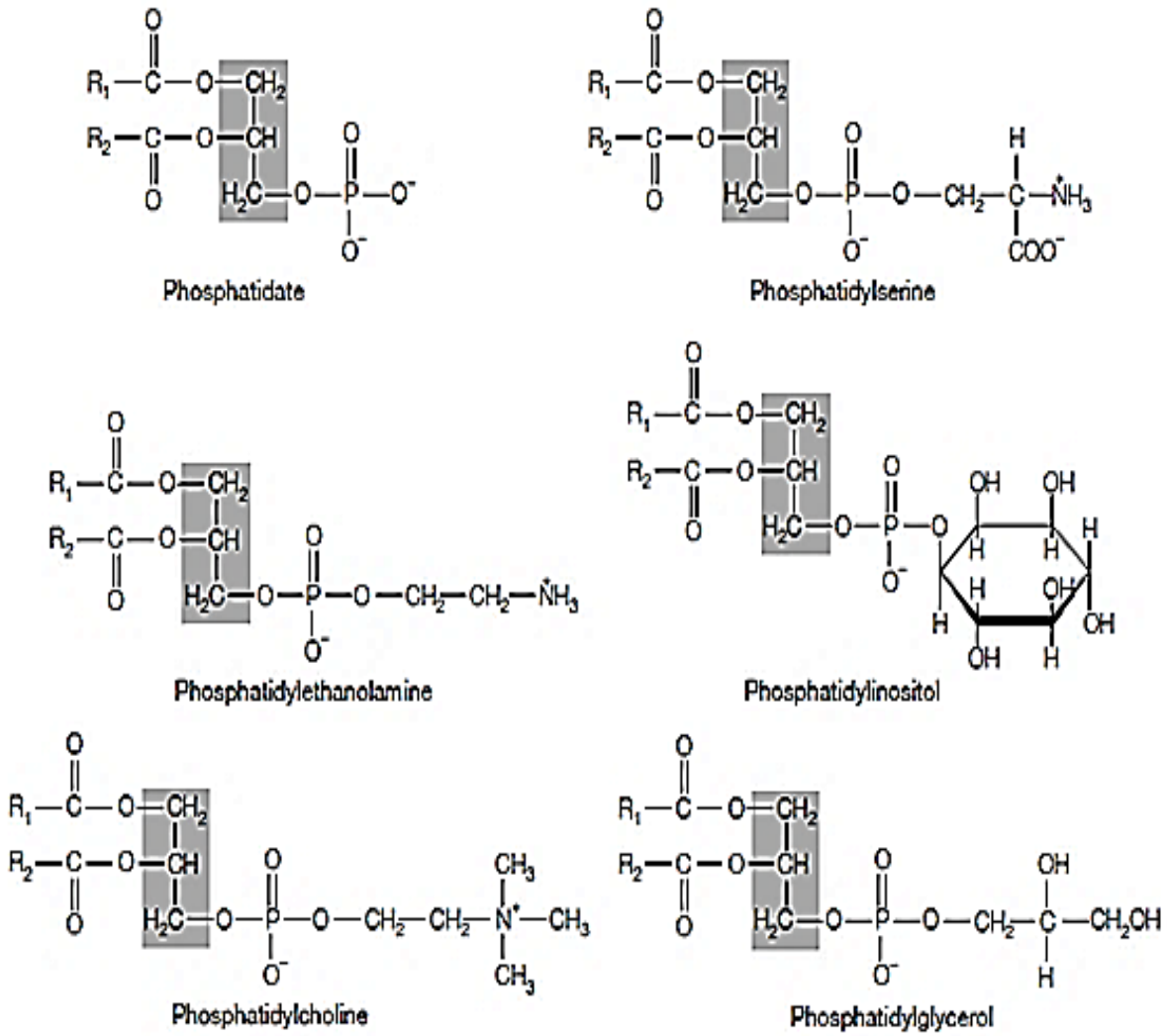

Figure 6. General structure of GP. R_1 and R_2 represent the fatty acyl chains esterified to glycerol backbone. Adapted from http://www.expertsmind.com/topic/membrane-lipids/glycerophospholipids-94413.aspx

Diphosphatidylglycerol or Cardiolipin (CL) is a dimeric PL with two phosphatidic acids linked by a central glycerol group. This group holds two more molecules of glycerol in its structure, and has four fatty acyl chains (Figure 7). The fact that the head group

alcohol is shared by two phosphates is a feature with important implications in CL mobility and conformational flexibility (35).

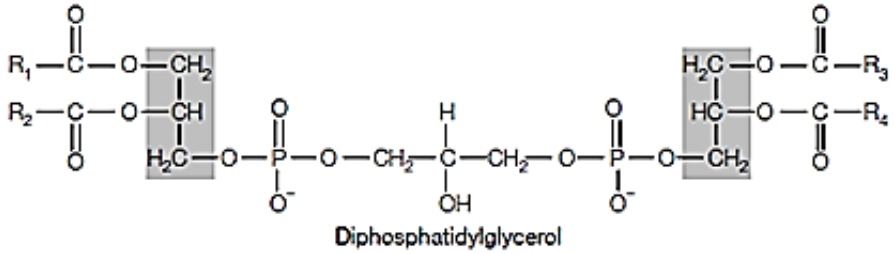

Figure 7. The general structure of cardiolipin, R1 and R2 represent the fatty acyl chains esterified to glycerol backbone. Adapted from http://www.expertsmind.com/topic/membrane-lipids/glycerophospholipids-94413.aspx

PLs can also be found in the membrane of organelles and where they are synthesized. PC, PI, PS and some PE are synthesized in the endoplasmatic reticulum while SM and some PC are synthesized in Golgi complex and CL and PE and are mainly synthesized in mitochondria (36).

Lysophospholipids are any derivative of a PL in which one or both acyl derivatives have been removed by hydrolysis and these could be: Lysophosphatidylcholine (LPC), Lysophosphosphatidic acid (LPA), Lysophosphatidyletanolamine (LPE), Lysophosphatidylinositol (LPI), Lysophosphatidylglycerol (LPG) and Lysophosphatidylserine (LPS) (35). Lysophospholipids act as extracellular mediators activating specific G-protein-coupled receptors (GPCRs) and some of them additionally play a role in intracellular signal transduction (37).The several types of PLs have influence on many proprieties of cellular membrane and the main properties affected will be discussed in the next subtopic.

PL that contain sphingosine backbone are called phosphosphingolipids. They have the same type of polar substituents as GP, mainly with choline polar head, differing only in hydrophobic group, which in this case is the ceramide, a molecule formed by the union of two FAs to a serine (which replaces the glycerol present in GP). The phosphosphingolipids more abundant in cell membranes are the sphingomyelin that is composed by a ceramide linked to phosphorylcholine polar head-group (Figure 8).

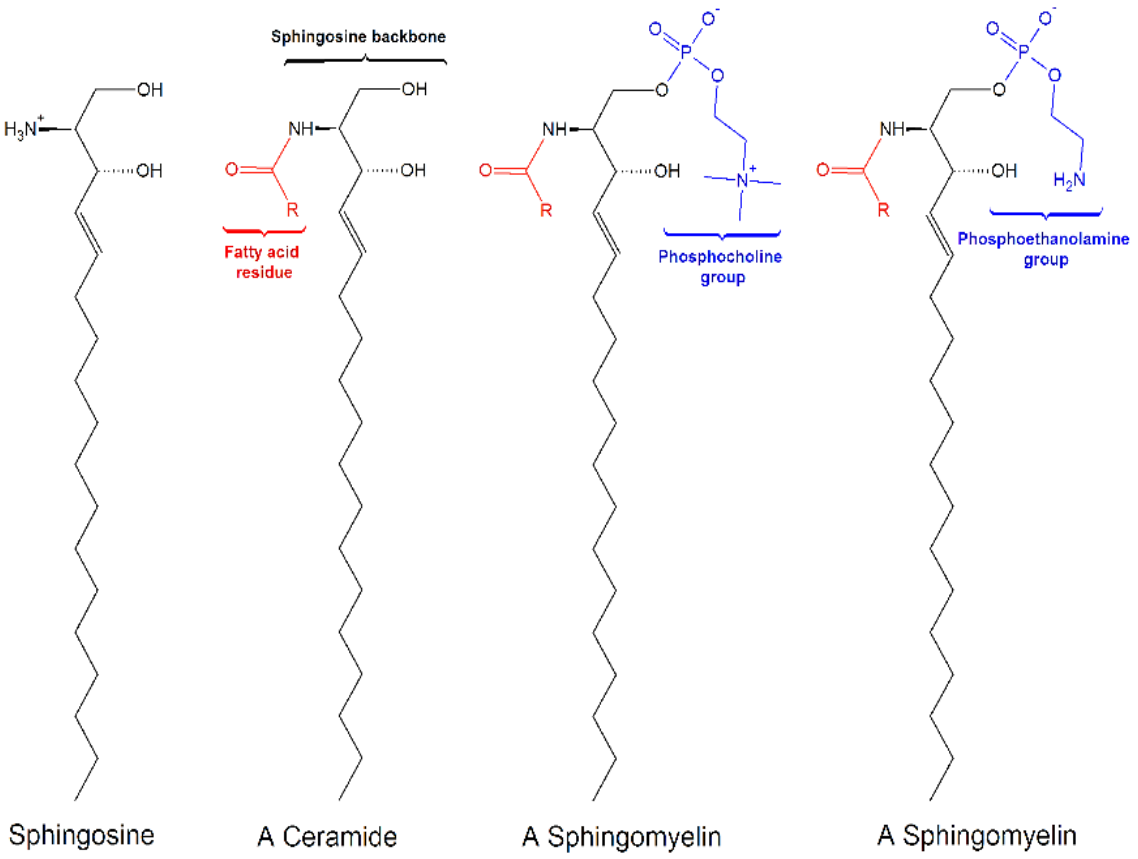

Figure 8. The general structure of Sphingomyelin. Adapted from http://en.wikipedia.org/wiki/File:Sphingolipids_general_structures.png

SMs and the glycosphingolipids (composed by ceramide linked to a sugar polar head) contribute to the formation of membrane microdomains (also termed lipid rafts) based on their interaction with CHL. Lipid rafts are regions of membranes with a distinct, characteristic structural composition and that appear to act as signaling platforms by bringing together various signaling components involved in intracellular signaling pathways, facilitating their interaction. The PLs present in these rafts regions are usually highly enriched in saturated FAs compared with the surrounding non-raft regions of the membrane. They allow a closer packing of lipids within rafts because the sphingolipids also contain saturated FA side chains, and CHL and saturated FAs are able to pack closely. Because these lipid rafts are less fluid and more organized than the surrounding membrane, many proteins involved in signal transduction are predominantly found in these domains. Furthermore, these liquid rafts appear to act as signaling platforms by bringing together various signaling components, facilitating their interaction (38).

3.2.1. Influence of the phospholipids structures on the membrane properties

PLs comprise the most abundant class of membrane lipids and they have fundamental significance for a large number of biological proprieties of membranes like fluidity, permeability, asymmetry and curvature. The membrane is a fluid structure, meaning that the components do not occupy definite positions and are susceptible to displacement or rotation dimensional translation. This fluidity depends essentially on the composition of PL bilayer and also on the presence of CHL. The size of the alkyl chains is crucial in the fluidity of biological membranes, since the greater the alkyl chains of the PL, the greater the interaction between them, making the membrane less fluid. Another important factor is the degree of unsaturation of those chains. A higher proportion of unsaturated bonds in the alkyl chain, will result in the increase of the distance between adjacent chains, an area associated with weaker interactions, and as a result the membrane becomes more fluid (39). Another determining factor in the fluidity of membranes is CHL. The CHL molecules are interspersed with PL in the membrane, thereby decreasing the interaction between alkyl chains. Therefore, CHL affects membrane fluidity preventing perfect packing between the long chain hydrocarbon. The membrane fluidity affects the cell's most essential functions, since it affects the rate of transport by transmembrane proteins and affects membrane permeability. The more fluid the membrane is, the more it becomes permeable to water and small hydrophilic molecules, and the greater the lateral diffusion of integral proteins (39,40).

Biological membranes, are characterized by a typical transversal asymmetry, maintained by differences in the lipid composition between the two leaflets with the exception of endoplasmic reticulum, where the lipids are transversal symmetrically distributed within the bi-layer (24).The aminophospholipids, PS and PE, are found primarily in the inner leaflet of the membrane, as well as PI and PA (less abundant lipids). Whereas the outer leaflet comprises the majority of lipids that contain choline, such PC and SM (35,41). The fact that the distribution of lipids in the bilayer is not homogenous, plays an important role in several functions such as maintain biophysical properties of specific membranes, facilitate membrane vesicle trafficking/fusion/budding and regulate activities of membrane proteins. An alteration of this PL asymmetry can also play an important role in activating cellular or biological processes like cytokinesis, blood

coagulation, cell fusion and recognition and removal of apoptotic cells (e.g externalization of PS by a cell) (24).

Each type of PL possesses the so called spontaneous curvature, which originates due the size of FA chains relative to the head group that defines the molecular shape of PL, which can influence the curvature of a membrane. In cases in which the molecule has a cylindrical shape, such as, PC and PS the curvature is not induced. Lipids with a small head group compared with the hydrophobic part, like PE, are cone-shaped while lipids with a hydrophobic part smaller than the headgroup, like LPC, has the shape of an inverted cone (24) (Figure 9). Both have influence in curvature and since the lipid compositions of the membrane leaflets are different, the whole bilayer may develop a curvature. These differences might have a physiological role in the generation of curvature, for example, during vesicle budding, and during membrane fusion (24,42).

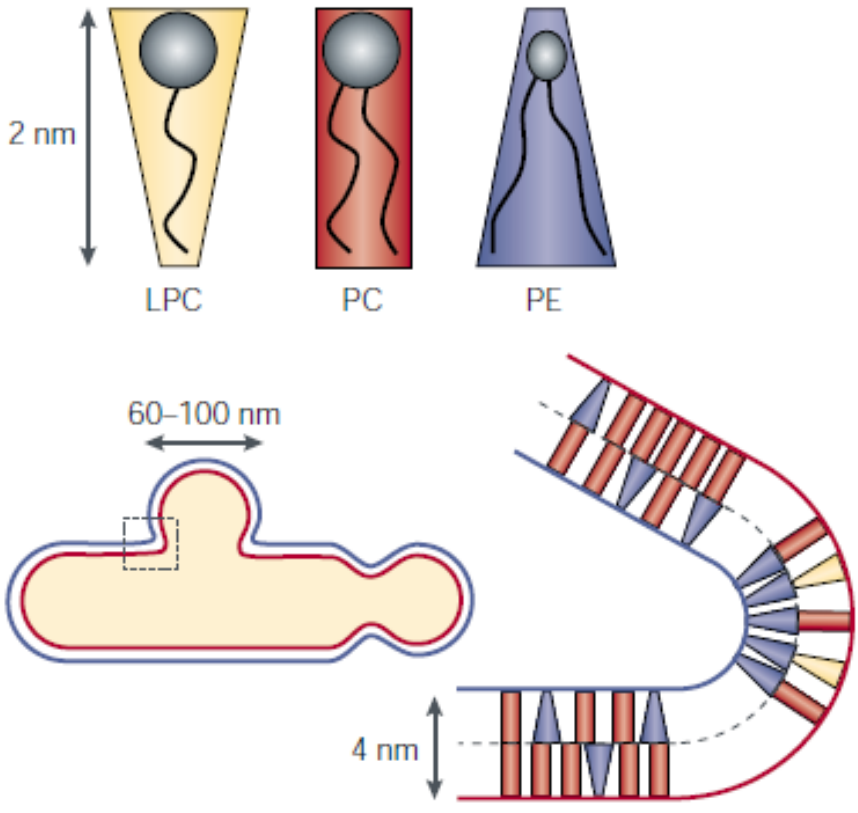

Figure 9. The molecular shape of PL depends on the relative size of its polar headgroup and apolar tails. Since the lipid compositions of the membrane leaflets are different it will influence the curvature of membranes. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine. Adapted from (42).

The selective permeability is another fundamental property of biomembranes influenced by the bilayer of PL. The semi-permeability is mainly due to the chemical nature of the biomembrane as well as the size and chemical nature of the substance which intends to charge through the membrane (43). Due to their hydrophobic nature of the hydrocarbon chains in lipid bilayers, they organize a virtually impenetrable barrier to the transport of ionic and polar substances. Specific membrane proteins regulate the movement of these substances into and out of cells.

3.2.2. Influence of PL on cell signaling

GP and sphingolipids also play an important role in signal transduction either by binding and/or activation of signal transduction molecules and as substrates of lipases, lipid kinases and phosphatases (44). Three kinds of enzymes, phospholipases, lipid kinases, and lipid phosphatases, produce most lipid-derived second messengers and three membrane lipids are the primary sources of these signaling molecules: phosphatidylcholine, phosphatidylinositol and its various phosphorylated derivatives, and sphingomyelin (45). These three types of PLs can be degraded by phospholipases to form molecules important for the signal transduction.

Phosphatidylcholine (PC) is the major membrane PL in eukaryotic cells and beyond that PC serves as a reservoir for several lipid messengers: it is the source of the bioactive lipids LPC, PA, diacylglycerol (DAG), LPA, platelet activating factor (PAF) and AA (28). The generation of these lipid messengers from PC depends on their metabolism. PC can be cleaved by three phospholipases: Phospholipase C (PLC) that cleaves the phosphorylated head group from PC and it leaves behind DAG in the membrane bilayer; Phospholipase D (PLD) that cleaves the polar head group from glycerophospholipid, producing phosphatidic acid that remains in the bilayer and releasing the soluble choline headgroup into the cytosol; phospholipase A_2 (PLA_2) that removes the sn2 FA, giving a free FA, released into the cytoplasm, and a lysophosphatidylcholine (LPC) (Figure 10) (28,45).

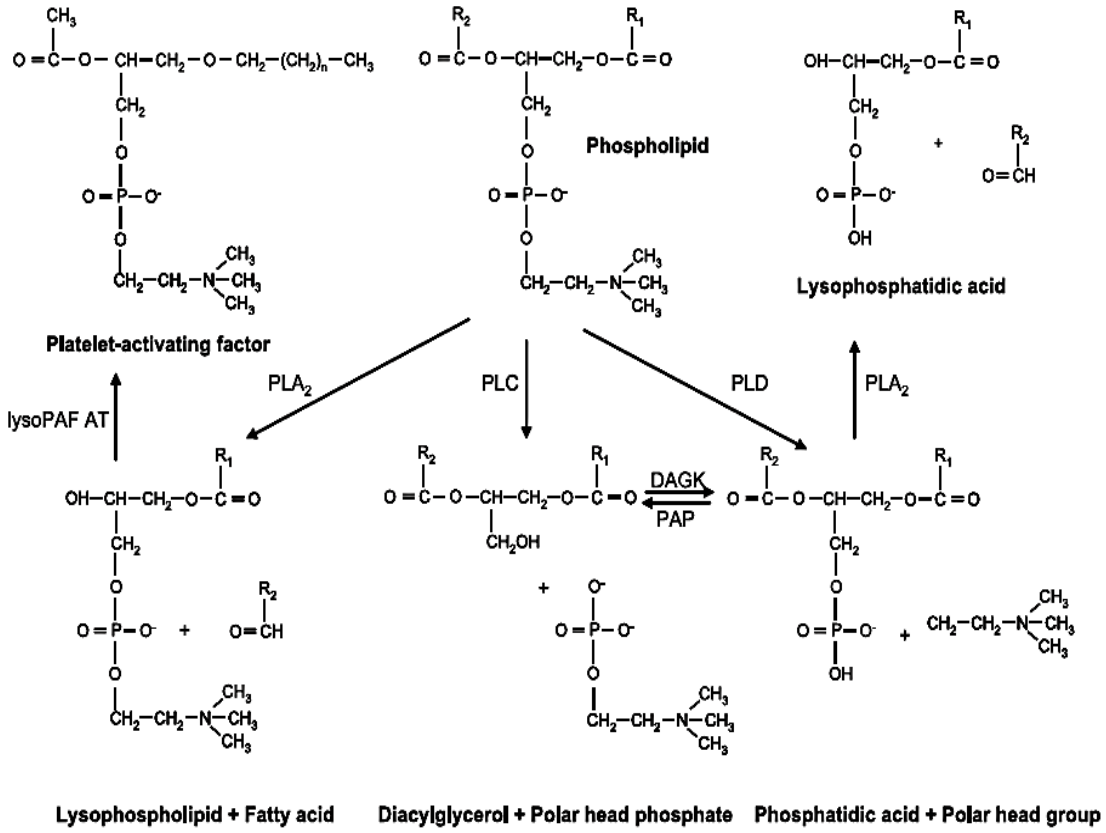

Figure 10. Cleavage products of the action of phospholipases on PLs. The specific parent PL shown here is phosphatidylcholine. R1 and R2, FAs; DAGK, DAG kinase; PAP, phosphatidic acid phosphohydrolase. Adapted from (45).

The phosphatidylinositol and its various phosphorylated derivatives are obtained by the addition of phosphate groups to phosphatidylinositol (by Lipid kinases) to make a variety of polyphosphoinositides, including phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2), and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Figure 11) (46). DAG and IP_3 are important signaling molecules produced by a family of phosphoinositide-specific phospholipase Cs, and their substrate is PIP_2 (28). DAG and phosphatidylinositol phosphate (PIP), are involved in calcium-mediated activation of protein kinase C and PI3K-AKT survival pathway. The lipid phosphatases can remove a phosphate from phosphatidic acid (another way to make DAG) and phosphates from inositol head groups (24,45).

Sequential action of two or more enzymes produces several other lipid second messengers. For example, cells can make DAG in two steps from phosphatidylcholine: PLD first makes phosphatidic acid (PA), and then PA is dephosphorylated to form DAG

by phosphatidic acid phosphatase (Figure 11) (28). Another example, is the action of PLD followed by PLA2 to yield lysophosphatidic acid (LPA) from phosphatidylcholine. LPA is a PL mediator with multiple biological functions such as, induction of cell proliferation, promotion of cellular tension and cell-surface fibronectin binding, and autocrine stimulation of platelet aggregation (47). When it is produced it escapes from cells and stimulate target cells by binding to G protein coupled receptors (GPCRs) and cells can respond to LPA in different ways: Activation of the PLC/IP3 pathway releasing the intracellular Ca^{2+} in some cells; activation of a mitogen-activated protein (MAP) kinase pathway stimulates cells division in some cases; and activation of Rho-family small GTPases stimulates formation of actin bundles in cultured cells. Platelet-activating factor (PAF), a modified PL also derived from PC, is formed by the cleavage of one FA from PC by PLA2 to yield LPC that by the action of an acetyltransferase is acetylated to yield PAF. PAF binds to the GPCRs that stimulate PLC to generate the two second messengers, IP_3 and DAG. These seconds messengers activate the PKC that can stimulates cell growth (45).

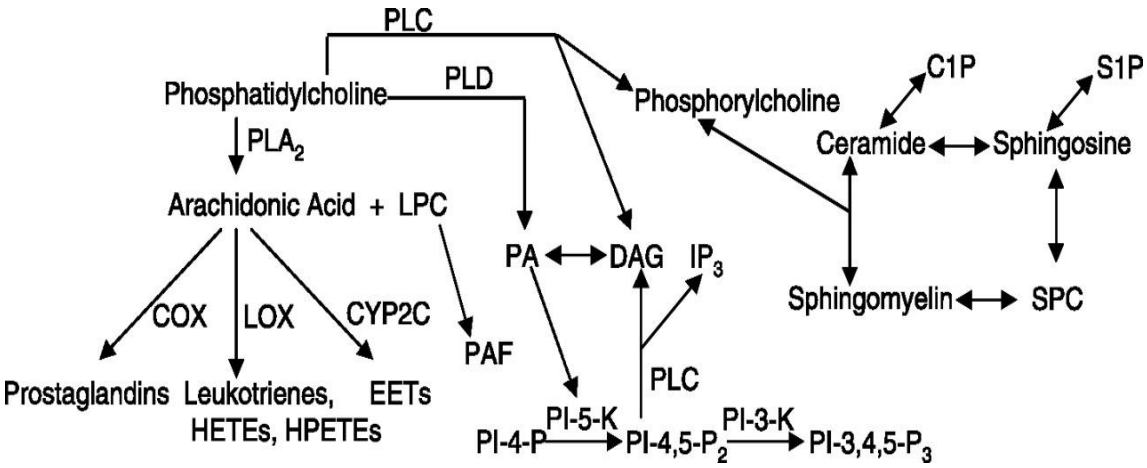

Figure 11. The interrelatedness and interconvertibility of the lipid signaling pathways. PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; LPC, lysophosphatidylcholine; COX, cyclooxygenase; LOX, lipoxygenase; CYP2C, cytochrome P-450 2C; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroxyperoxyeicosatetraenoic acid; EETs, eicosatrienoic acids; PAF, platelet-activating factor; PA, phosphatidic acid; DAG, diacylglycerol; IP_3, inositol (1,4,5)-trisphosphate; PI(4)P, phosphatidylinositol (4)-phosphate; $PI(4,5)P_2$, phosphatidylinositol (4,5)-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol (3,4,5)-trisphosphate; PI-5-K, phosphatidylinositol 5-kinase; PI-3-K, phosphatidylinositol 3-kinase; C1P, ceramide- 1-phosphate; S1P, sphingosine-1-phosphate; SPC, sphingosylphosphorylcholine. Adapted from (45)

Sphingomyelin can originate the sphingosine and sphingosine-1-phosphate (S1P) by a succession of enzymatic reactions (48). Ceramidase removes the FA, and sphingomyelinase removes the phosphorylcholine head group to form sphingosine. Then sphingosine kinase adds phosphate to C1 to make sphingosine-1-phosphate (Figure 12). Growth factor signaling pathways might regulate this lipid kinase. S1P escapes from cells and transmits signals through a family of G-protein-coupled receptors. Cells respond by releasing Ca^{2+}, which influences motility and growth, as well as smooth muscle contraction (37,49).

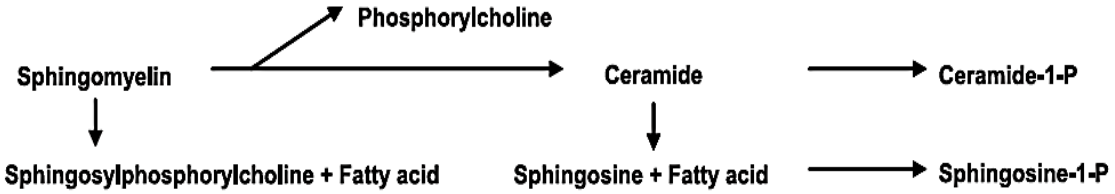

Figure 12. Structures released from sphingomyelin by enzymatic cleavage. Adapted from (45).

Several important cellular processes like cell proliferation, metabolism apoptosis and migration are controlled by signaling lipids such as phosphoinositides, eicosanoids, sphingomyelin and FAs. Enzymes that modify this lipids like phospholipases, phosphoinositide-3-kinase, COX, LOX, sphingosine kinase and sphingomyelinase are controlled by extracellular signals from growth factors, cytokines and nutrients. These enzymes and their targets constitute a complex lipid signaling network and imbalances in this network seems to contribute to the pathogenesis of human diseases such as breast cancer (44).

4. Changes in lipid profiling and breast cancer

Lipids are nowadays recognized as metabolites that have a key role in the development of several diseases and cancer is no exception. In addition, the lipid composition might vary with the diet that contains different types of FAs and many studies have tried to determine whether SFA, MUFA and PUFA have different effects in

the development and progression of breast cancer. Studies with cell culture have found that SFA (50), MUFA, TFA (32) and n-6 PUFA promote BC cell growth (51,52), whereas ALA, EPA and DHA inhibit BC cell growth (52). An exception within the MUFA class is oleic acid (18:1n9), which has been observed to have anti-tumorigenic effects by suppressing overexpression of HER (52,53).

In animal studies, SFA and n-6 PUFAs, have a strong mammary tumor-enhancing effect (54,55) . In human studies of SFA and n-6 PUFA, the results are contradictory and it is not possible to reach a conclusion. At the same time, many studies support a role for n-3 PUFA in the prevention or inhibition of breast cancer. n-3 PUFAs can have chemopreventive and chemosuppressive properties against breast cancer and can be auxiliary agents for cancer therapy (56–58). Studies in human populations are limited, some have pointed out that high consumption of n-3 PUFAs is related to a reduced risk of breast cancer. Moreover, other studies failed to find a significant association. However, a large body of research involving animal and cell culture models supports a role for the long-chain n-3 PUFA (EPA and DHA) in the inhibition of tumor growth. Studies suggest that n-3 PUFAs induce modifications of membrane structure and function of breast cancer cells by increasing the degree of unsaturation of PLs (58). Thus, epidemiological evidence combined with the demonstrated effects of n-3 PUFAs on cancer in animal and cell culture models, has motivated the development of clinical interventions using n-3 PUFAs in the prevention and treatment of cancer.

So, while most of the results obtained with DHA and EPA show a decrease of tumor cell proliferation and/or aggressiveness, there is some evidence that AA may have opposite effects. It is thought that having a high amount of n-6 PUFAs and a low amount of n-3 PUFAs in the diet, results in a high n-6/n-3 ratio and contributes for breast cancer. This shows that the ratio of n-6 to -3 may be more important than the absolute amount of n-3 PUFAs, as suggested by animal and human studies (59–61). So it seems that the development of dietary strategies that will result in an increase of n-3 in the diet, blood and tissues, associated with decreased omega-6, might have a beneficial effect in the health.

Given all the results presented, it is clear that there is a very likely association between the dietary FA and breast cancer.

Lipid rafts are known to be rich in signaling molecules and to regulate signal transduction in normal and cancer cells. Enrichment of lipid rafts with n - 3 PUFA are able to modify lipid raft biochemical and biophysical features. This will lead to the

decrease of breast cancer cell proliferation, probably through different mechanisms related to acyl chain length and unsaturation. FA are incorporated in breast cancer lipid rafts with different specificity for the PLs, in particular PUFA are incorporated in PS, PI and PC. While EPA may contribute to cell apoptosis mainly through decrease of AA concentration in lipid raft PLs, DHA may change the biophysical properties of lipid rafts leading to a redistribution of key proteins (38,62).

Diverse evidences of the probable mechanisms regarding how PUFA may modify the carcinogenic process, have been proposed. FAs intake by the cell, regulates lipid metabolism, including FA elongation and desaturation. Thereafter, FAs are esterified to glycerol and enter the PL biosynthetic pathway. Therefore, the kind of FA in the diet may influence the PLs of the cell membrane and thus affect cell properties and function. A high content of PUFA makes the cell membrane more fluid, which will result in alterations of membrane fluidity that can seriously affect functional properties of the cell and induction of apoptotic pathways, resulting in cell death (58,63). Another mechanism by which the fatty diet can interfere in the process of carcinogenesis is by lipid peroxidation. PUFAs are susceptible to lipid peroxidation and as such, PUFA in the diet can result either in an increased consumption of oxidized FAs, or promote lipid peroxidation in the body. The metabolites of this reaction are extremely toxic and may promote carcinogenesis through stimulation of cell proliferation. Also the dietary FAs and their metabolites may affect the activity of transcription factors, gene expression and signal transduction, mechanisms that generate changes in proliferation or apoptosis (64).

Finally, the proportion of PUFA in the cell membrane is one of the main factors that will determine the class of eicosanoids that will be synthesized (28). Eicosanoids are biologically active substances derived from essential FA that act in the body by modulating the inflammatory activity and immune response, and are involved in platelet aggregation, cell growth and differentiation (28,64,65). Further studies are needed to evaluate and verify these mechanisms in humans to have a better understanding regarding the effects of PUFA intake on cancer risk/ development.

FAs are not the only lipids connected to breast cancer physiopathology (66). As mentioned above, PLs are also involved in signal transduction and are thought to be involved in cancer. It is known that alterations in PLs are found in breast cancer cells and these alterations can change the normal function of the cell (66,67). Some studies showed high levels of PC and PE were detected in breast cancer cells and contain considerably greater amounts of unsaturated FAs when compared to normal breast tissue (68).

However a recent study by Doria *et al* that described the differences and similarities between mammary epithelial and breast cancer cells, and between breast cancer cells with different levels of aggressiveness found different changes. The most abundant PL class in all cell lines was PC, followed by PE. Increased relative amounts of LPCs were found in more aggressive, indicating that alterations in these PLs could be associated to malignant progression. Also changes in PE profile also were found, PE relative levels were highest in non-malignant cells (67).

PIs and their metabolites such as phosphatidic acid (PA) and DAG, and phosphorylated derivatives of PI, phosphoinositides (PIP), are very important contributors to cellular signaling cascades that activate proliferation, maintain survival and promote migration (69). Imbalances in their relative levels lead to changes in cellular functions that may contribute to the onset of cancer (70).

S1P is a signaling molecule that has the ability to act as an intracellular second messenger, as well as an extracellular stimulus through specific G protein-coupled receptor (GPCRs) (48). S1P regulates many processes important for the normal cell physiology (46,71). Alterations in S1P signaling, as well as in the enzymes involved in its synthesis and metabolism, have been observed in many types of pathological conditions including in breast cancer (72). LPA, is a lysophospholipid which has some structural similarities to S1P, performing many of its extracellular effects acting in specific G-protein-coupled receptors (46). These interactions are important for the cell proliferation, migration and survival. Therefore deregulation of LPA production, receptor expression or signaling will probably contribute to cancer initiation, progression and metastasis (37).

The Lipids reported above have an essential role in normal function of the cell. Changes in lipidome of cells can lead to imbalances in biological properties of membranes and on signaling that can lead to onset and development of cancer. It is known that different dietary FA have different effects, however little is known about their impact in lipidome of breast cancer cells and their association with cell growth, viability and apoptosis. Thus, the role of different dietary FAs in the profile of PLs of cells of breast cancer is not completely understood and remains controversial, being necessary more studies to reach more conclusions.

5. Lipidomics approach on breast cancer

Lipidomic is considered a branch of metabolomics that studies the lipidome, i.e., the total lipid composition of a cell or organ, and their interactions with other lipids, proteins and other molecules present in biological samples in their natural environment. Lipidomics have been subject of a great development in the last decade (73).

There are several experimental approaches that could be used in lipid research, such as chromatography, mass spectrometry, nuclear magnetic resonance (NMR). NMR could be used to analyze lipids directly in a nondestructive manner, however, the sensitivity of NMR is low and allows to identify the more abundant and dominant lipids (such as CHL and PC). Having this into consideration, the most used techniques nowadays are chromatography and mass spectrometry (74).

Lipidomics usually involves several steps. First is necessary the extraction of lipid from tissues or cells. Almost all lipid extraction methods use organic solvents to take advantage of the high solubility of lipids in organic solvents, i.e., lipid separation is created between immiscible solvents because lipids go into a hydrophobic phase. Then, to separate class of PLs it is used chromatography methods, and the most popular and simple is thin-layer chromatography (TLC), this facilitates further analysis and identification of lipids, already divided by their families. The components of the sample will be separated based on their varying physical and chemical properties, imparting different affinities for the two phases allowing the separation and identification of different PL classes be comparison to the standards also applied on the TLC plate (75). PL content of each class can be measured by phosphorous assay that measures the inorganic phosphate present in the lipid sample.

To study FA profiling, mass spectrometry coupled to gas chromatography (GC-MS). Mass spectrometry is a technique that performs the molecular identification through determining the ratio of mass to charge (m/z) of a molecular (74,76,77). A mass spectrometer typically is constituted by four principal components: a system to introduce the sample into the source of ionization; the ionization source, where the analyte molecules enter the instrument and are transformed into gaseous ions; the mass analyzer that separates the ions according to their mass-to-charge ratio (*m/z*); and the detector in which the separated ions are collected and characterized by the production of a signal whose intensity is related to the number of detected ions (78).

With the rapid development of lipidomics techniques, more attention has been paid to lipids because of their important roles in tumor diagnosis and therapy. MS has played an essential role in the study of PL and fatty acids in cancer cells, because it is a technique that combines speed, specificity, selectivity and sensitivity (67,74). It is known that a lipidomic approach is useful to define differences among PL classes and their molecular species. However, until this moment information about lipidome changes in cancer progression is rare, because in general these studies are only centered on one or few PL classes. Therefore, it is necessary to carry out more studies to provide more information (79,80).

Studies of effect of different dietary FAs on lipidome of cancer cells are scarce. Understand the role of dietary FAs in the profile of lipids of breast cancer cells and correlate it with cell proliferation and apoptosis is very important to prevention, and because it could provide new therapeutic opportunities and biomarkers for breast cancer that could modulate the progression of breast cancer.

6. Aim of the study

The role of different dietary FA in breast cancer cell growth is not completely understood and remains controversial. Further, the relationship between cell proliferation, death and how these processes are related to changes in the cellular lipid profile remains unknown. Thus, lipidomic studies are needed to better understand these issues.

The aim of this work was to establish how the lipidome of a breast cancer cell line differs with exposure to dietary FAs and correlate these findings to the effects in proliferation and apoptosis. For this purpose, the breast cancer cell line T47-D was cultured with oleic acid (C18:1n-9) or linoleic acid (C18:2-n6) for 6 days, following which viability, apoptosis, TG and CHL content, PL classes and FA changes were analyzed.

II. Methodology

II. Methodology

1. Chemicals

PE, PS, PC, PI, SM, LPC and CL standards (Avanti Polar Lipids), triethylamine (Acros Organics), chloroform and n-hexane (VWR Chemicals HPLC grade), methanol (Fisher Chemical HPLC grade), ethanol (Panreac), primuline (Sigma-aldrich), Mili-Q water (Millipore Synergy), methyl-tert-Butyl ether (MTBE) (VWR Chemicals *HPLC grade*), ammonium molibdate de amónio (Panreac), quantification standard of PL (Riedel-deHaen), acetone (Sigma-aldrich *HPLC grade*), boric acid (BDH Chemicals), percloric acid 70% (Chemlabs *HPLC grade)*, sodium chloride (NaCL) (Absolve), potassium hydroxide (KOH) (EKA Chemicals), TLC silica gel 60 plates with concentration zone (2.5x20cm) were purchased from Merck (Darmstadt, Germany), FBS (PPA, Austria), Trypsin-EDTA, PEST and PrestoBlue™ reagent (Invitrogen, Life Technologies, USA). Insulin, Triton X-100 and DMSO (Sigma, USA). For protein quantification it was used the DC protein kit (BioRad, Hercules, CA) and for TG and CHL quantification it was used a LiquickCor-TG and LiquickCor-CHL kit (Cormay, Poland), respectively. Finally for apoptosis assay it was used CellEvent Caspase-3/7 Green Detection Reagent (Invitrogen, Life Technologies, USA).

2. Cell culture

T47-D cells were grown in RPMI-1640 medium plus PEST and 10% FBS. For lipidomic and immunoblot experiments cells were seeded in 10 cm-diameter dishes and for cell survival, apoptosis and cell counting experiments 1000 cells / well were seeded in 96-well plates. Twenty-four h later, medium was changed to RPMI-16040 + PEST + 2% FBS + 5 ug/ml insulin with addition of OA or LA dissolved in DMSO or same volume of control DMSO alone. Control group received same volume of DMSO (final dilution1/500).

3. Cell viability

T-47D cell viability was measured using PrestoBlue™ reagent. Briefly, the cells treated with the FAs were incubated with the reagent directly added to the cells in culture medium diluted 100x. Then, cells were incubated for 240 minutes at 37º C and the absorbance was measured at 570 nm and 600 nm, every half hour, using a microplate reader (Multiscan 90, ThermoScientific). The percent reduction of PrestoBlue reagent were calculated from the absorbance data by using the following equation: $\frac{(\boldsymbol{O2}\times\boldsymbol{A1})-(\boldsymbol{O1}\times\boldsymbol{A2})}{(\boldsymbol{R1}\times\boldsymbol{N2})-(\boldsymbol{R2}\times\boldsymbol{N1})}\times 100$, where: O1=molar extinction coefficient of oxidized PrestoBlue reagent at 570 nm (80586), O2=molar extinction coefficient of oxidized PrestoBlue reagent at 600 nm (117216), R1= molar extinction coefficient of reduced PrestoBlue reagent at 570 nm (155677), R2= molar extinction coefficient of reduced PrestoBlue reagent at 600 nm(14652), A1=absorbance of test wells at 570 nm, A2=absorbance of test wells at 600 nm, N1=absorbance of media only wells at 570 nm and N2=absorbance of media only wells at 600 nm. Two independent experiments were performed, using at least triplicates.

4. Lipid extraction

Following growth with the FAs for 6 days, cells were scrapped using a rubber scraper and re-suspended in 1 ml of PBS. From this solution, 100 µl were separated for protein quantification. The remaining 900 µl were used for lipid extraction with methyl-tert-butyl ether (MTBE) based protocol for lipid extraction (81). Briefly, 1,5 ml of methanol was added to the cell pellet and the solution was vortexed. Then, 5 ml of MTBE was added and the mixture was incubated for 1 h at room temperature. Separation of phases was achieved by adding 1.25 ml of mili-Q H_2O water. After 10 min of incubation at room temperature, the sample was centrifuged at 1500 rpm for 10 min. The upper phase (organic) was collected, and the lower phase was re-extracted with 2 ml of MTBE/methanol/water (10:3:2.5, v/v/v). Both organic phases were dried in a speed vacuum centrifuge. Extracted lipids were dissolved in chloroform and then dried under nitrogen flow for storage at -20ºC.

5. PL quantification

To quantify the total amount of PLs as well as amounts of each PL class separated by TLC, a phosphorus assay was performed according to Bartlett and Lewis (82). Briefly, 0.65mL of percloric acid 70% were added to the samples which were then incubated in a heating block, 1 hour at 200°C. Standards were prepared by the addition of 0.65mL of percloric acid 70% to aliquots with 0.1 to 2 µg of phosphate. To all samples and standards, we added 3.3 mL of H_2O, 0.5 mL of ammonium molibdate (2.5 g ammonium molibdate/100 mL of H_2O) and 0.5 mL of ascorbic acid solution (10 g ascorbic acid/100 mL of H_2O), and vortexed after the addition of each solution, following by the incubation in a bath at 100ºC, 5 min. The absorbance was measured at 800 nm, in a microplate reader (Multiscan 90, ThermoScientific). In the case of PLs separated by TLC, prior to spectrophotometric determination, the samples were centrifuged 5 min at 4000 rpm to separate PLs from silica. The phosphorous content in each spot was related to total phosphorous content in the sample. Three independent experiments were performed, using duplicates for each experiment.

6. Thin layer chromatography

PL classes from the total lipid extract were separated by thin TLC using silica gel plates with concentrating zone 2,5x20cm. Before the separation, the plates were washed in a solution of $CHCl_3$: MeOH (1:1 v/v) and treated with a solution of boric acid in ethanol (2.3% w/v). Thereafter, the plate were dried in an oven at 100ºC during 15min and then 20 µl of each total lipid extract re-suspended in chloroform and containing about 30 µg of total PL (in a concentration of 150 µg of PL per 100 µl) were applied in the plates. The plates were developed with a mixture of solvents $CHCl_3$: MeOH: H_2O: triethylamine (30:35:7:35, v/v/v/v). To visualize the PL spots after elution, the TLC plates were stained with a primuline solution (50 µg/100 mL acetone: water 80:20, v/v) and visualized with a UV lamp (λ=254nm). After the identification of the PL spots, by comparison with PL standards, the spots were scraped to quantify amount of phosphorus with a phosphorus assay to calculate the percentage of each PL class in the total amount of PL in the sample. Three independent experiments were performed, using duplicates for each experiment.

7. Protein quantification by DC assay

For protein quantification we used the DC protein kit. We added 20 µL of S reagent from the kit to 1mL of reagent A (alkaline cooper tartrate reagent), to form reagent A'. Standards of BSA 0.2 mg/mL to 1.5 mg/mL were also prepared. Then, 5 µL of each standard and each sample were mixed with 25 µL of reagent A' and 200 µL of reagent B (a dilute Folin Reagent), and incubated at room temperature for 15min. The absorbance of all the samples was measured at 750 nm using a microplate reader (Multiscan 90, ThermoScientific). Three independent experiments were performed, using duplicates for each experiment. Protein concentration was used to normalize the data and compare the amount of TGs, CHL and total PL between the treatments.

8. Gas chromatography coupled with mass spectrometry

To analyze the FAs present in total lipid extract we used GC-MS. We used 20µL of each total lipid extract, containing about 30 ug of total PL, and dried it with a nitrogen flow. The sample were re-suspended with 1mL of hexane and then mixed with 200µL of KOH (2M). As the next step, 2 mL of NaCl were added and the mixture was then centrifuged at 2000 rpm during 5 minutes. The upper phase was transferred to another tube and the solvent was evaporated under a nitrogen flow. Finally, the sample was re-suspended on 60 µL of n-Hexane, and 2 µL of this solution was injected in a gas chromatography-mass spectrometry (GC-MS). The GC was an Agilent Technologies 6890N Network (Santa Clara, CA) equipped with a DB-1 column with 30 m of length, 0.25 mm of internal diameter, and 0.1 µm of film thickness (J&W Scientific, Folsom, CA) and was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range m/z 40-500 in a 1 s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 40 ºC, standing at this temperature for 0.5 min, a linear increase to 220 ºC at 20 ºC/min, followed by linear increase at 2ºC/min to 240ºC, and then at 5 ºC/min to 250 ºC. The injector and detector temperatures were 220 and 230 °C, respectively. The gas used was Helium at a flow rate of 1.7 mL/min. Identification and analysis of peaks of the spectrum was carried out using a using software that came with the equipment (MSDA). The peaks were identified by the retention time and using a standard library.

Then the peaks were integrate to obtain an area. Each area was related to total area of the sample giving the % relative content of FAs. Three independent experiments were performed, using duplicates for each experiment.

9. Total triglyceride and cholesterol

Prior measurement, we prepared the samples to allow the formation of liposomes needed for the quantification using this type of colorimetric kit. For this, we used 20 µL of sample previously dried under nitrogen flow, which was re-suspended in 40 µL of ammonium hydrogen carbonate 5 mM pH 7.4 buffer with addition of 5 % of Triton X100. Then, the sample was sonicated for ten minutes to emulsify and used for TG and CHL quantification. Total TG quantification was carried out with the LiquickCor-TG kit. Briefly, for the working reagent preparation we mixed gently 4 parts of 1- TG with 1 part of 2-TG reagents. This solution (0.5 mL) was mixed with 5µL of each sample and the standard, and incubated at 37ºC, 5 min. At last, the absorbance of all the samples was measured at 500 nm using a microplate reader (Multiscan 90, ThermoScientific). For total CHL quantification we used the LiquickCor-CHOL kit. Briefly, 5µL of each sample and the standard were mixed with 0.5 mL of reagent, and incubated at 37ºC, 5 min. The absorbance of all the samples was measured at 550 nm using a microplate reader (Multiscan 90, ThermoScientific). Three independent experiments were performed, using duplicates for each experiment.

10. Cell counting

To count the cell number resulting from 6 days of treatment with the FAs, cells were detached with Trypsin-EDTA and counted in a TC 20 ™ Automated cell counter (BioRad). All treatments were done with four replicates.

11. Apoptosis assay

For detection of apoptosis we used the CellEvent Caspase-3/7 Green Detection Reagent which is a fluorogenic substrate for detection of activated caspases 3 and 7 in apoptotic cells. It was prepared by adding 1 drop of reagent in 500 uL of PBS and then 100 uL of this solution was added to each sample followed by incubation for 1 hour at room temperature in the dark. To evaluate apoptosis it was used an inverted microscope (Niko TiU) using a 480 nm FITC filter.

12. Statistical Analysis

We analyzed three independent experiments (n=3). The results were presented as means ± standard deviation (SD) or standard error of mean (SEM) when more than one independent measurement was carried out for the same sample. Differences between the groups were compared by one way analysis of variance (ANOVA) with the Dunnett's post-hoc test. For comparison of two groups two-tailed *t*-test was used. Values of $p<0.05$ were considered statically significant. Statistical analysis was carried out using PRISM GraphPad Software, Inc.

III. Results and Discussion

III. Results and discussion

As one approach to understand the influence of dietary MUFAs and PUFAs on human breast cancer, we have studied the effects of OA and LA *in vitro* in cultured human breast cancer cell line, T-47D (belongs to the luminal A group, with low metastatic potential). We chose to use a concentration of 1ug/mL C18:1n9 (Oleic acid –OA), previously shown to exert antiproliferative effects (83); and concentrations of 1ug/mL C18:2 n-6 (linoleic acid - LA1) and 2ug/mL C18:2 n-6 (LA2), previously shown to be pro-proliferative (84).

The lipid changes in lipidome induced by the presence of these FAs supplementation were study through a lipidomic analysis, based on techniques such as TLC and GC-MS. TLC allowed the separation of PL classes and GC –MS allowed the separation of FAs and their quantification. TGs and CHL were quantified by colorimetric methods. Cell viability and apoptosis were also evaluated.

1. Cell viability

On a first approach we did an assay of cell viability commonly used to evaluate cell metabolic activity (Figure 13).

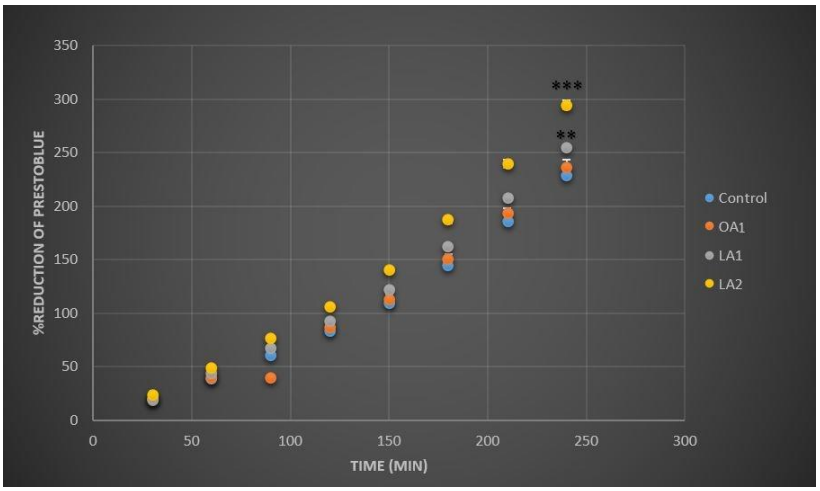

Figure 13. Effects of FAs supplementation on cell viability. Cell metabolic activity was determined using PrestoBlue assay for cells treated with different FAs and metabolic activity was measured every half hour until 240 minutes. Legend: Control – Without FA; OA1- Oleic acid (C18:1n9 1ug/mL); LA1- Linoleic acid (C18:2 n-6 1ug/mL); LA2- Linoleic acid (C18:2 n-6 2ug/mL). Statistical significance was measured with One-way ANOVA and Dunnett's post-hoc test, **: $p < 0.01$ (LA1); ***$p<0.001$ (LA2) starting in 180 min.

This assay is based on detection of metabolic activity underlying resazurin reduction. Resazurin is a fluorescent redox indicator dye that is frequently used to determine viability of cells directly in culture, where only viable cells are able to convert the dark blue oxidized form of the dye (resazurin) into a red-fluorescent reduced form (resorufin). The results are expressed in % of reduction, so a biggest reduction is correlated to a greater total metabolic activity that is used quantitatively as a measure of cell growth and integrity. Thus, we can see that cells treated with LA1 (**: $p<0.01$) but especially with LA2 (***: $p<0.001$) showed increased reduction of resazurin into resorufin over time particularly since the 180 min. This indicates that a medium with LA increased stimulation of T47-D metabolic activity, being more effective in supporting cell growth than the treatment with OA that had approximately the same values of control.

The results were consistent with literature, which showed that a range of concentration of LA had a stimulatory effect on the growth of T-47D cells, including 1 and 2 ug/mL of LA. Only at concentrations exceeding 10 ug/mL was observed cytotoxicity in breast cancer cells (84).

To confirm the viability assay results, cells were counted and apoptosis was evaluated. For this new set of experiments, T-47D cell line was treated with the same FAs (OA1, LA1 and LA2) but this time, an additional condition has been added, a culture medium with (2ug / mL OA) in order to see if the lack of variation in metabolic activity with OA was related to suboptimal OA concentrations.

2. Cell counting

Cell counting results are presented in Figure 14.

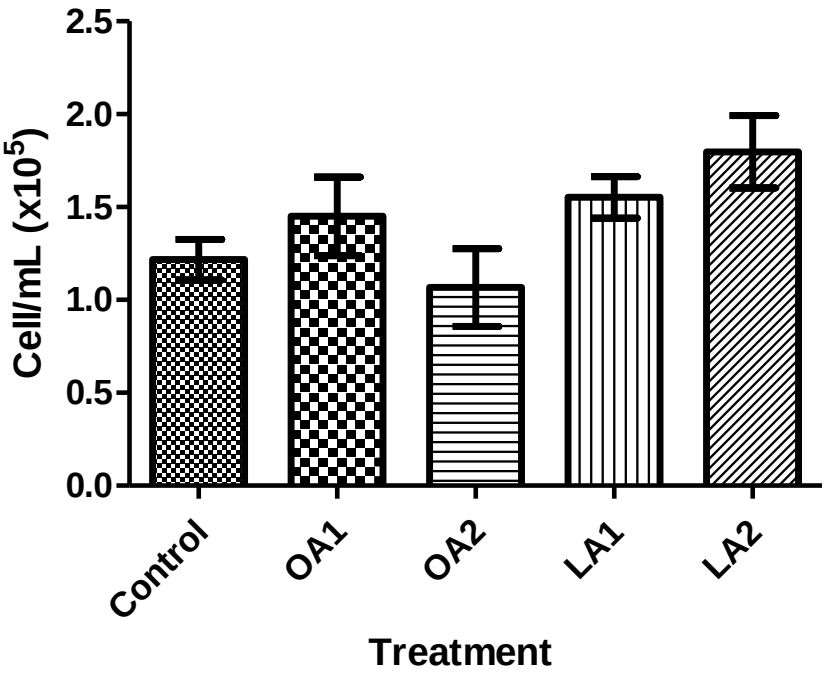

Figure 14. Cell number was measured by cell counting. The graph shows the number of cells per ml in control and treated with OA1, OA2, LA1 and LA2. Each value represents the average of four measurements. Statistical differences were analyzed with One-way ANOVA and Dunnett's post-hoc test, but no differences were found.

There was no statistically significant difference between the numbers of cells treated with these FAs. However, a clear tendency to increase cell number was observed for LA2 in relation to other treatments. In addition to this, we can see a slight decrease of cell number in the treatment with OA2. These results suggest that LA2 could enhance cell population growth and OA2 might have the opposite effect.

3. Apoptosis assay

To evaluate apoptosis, it was used the CellEvent Caspase-3/7 Green detection reagent, that is a new fluorogenic substrate for activated caspases 3 and 7, showing bright green nuclei when caspase 3/7 are activated. Therefore, this assay showed qualitatively which of the treatments promote a greater apoptosis. In Figure 15 the results of the assay

are shown and the treatment with OA2 is the one which resulted in more apoptosis comparatively to control.

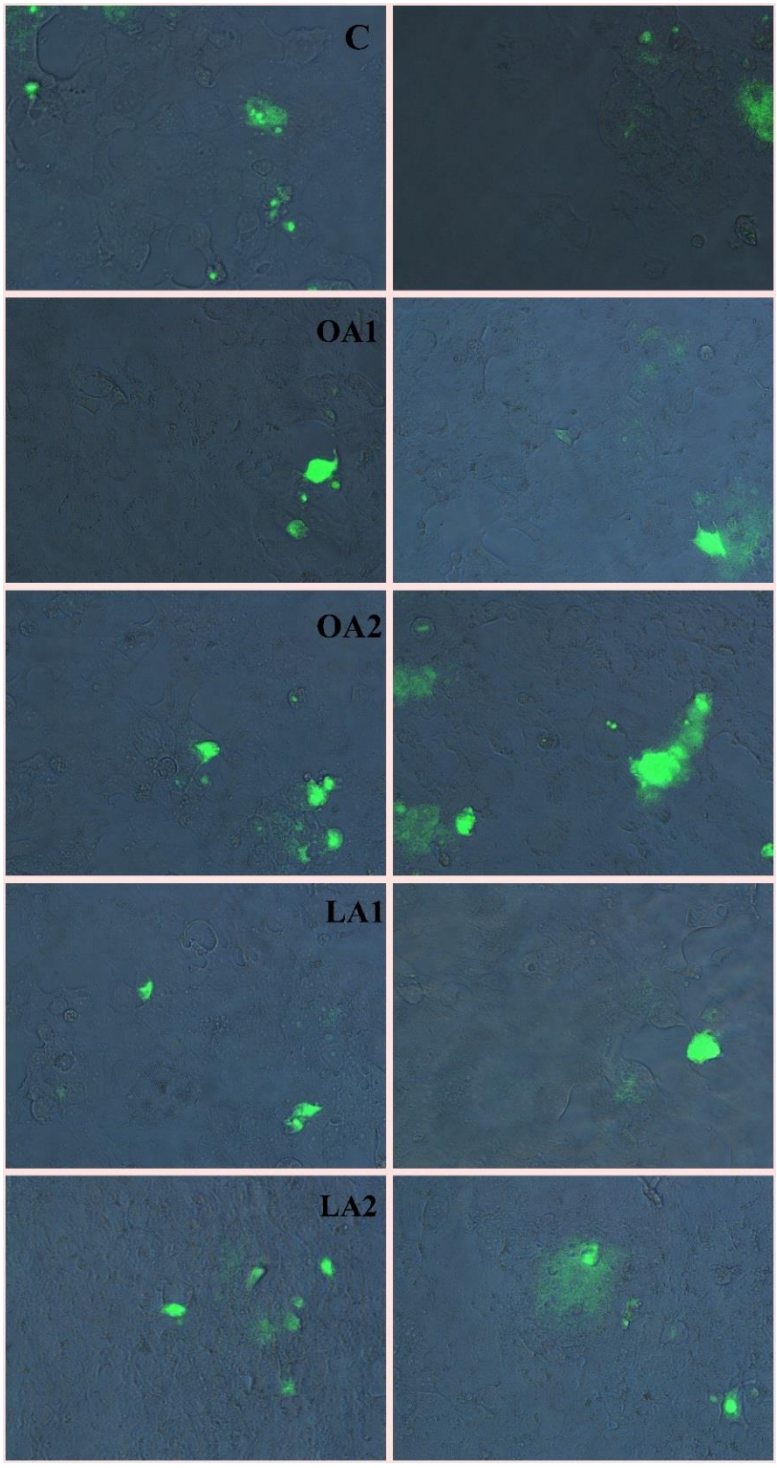

Figure 15. Apoptotic assay of T-47D cells treated with OA and LA in different concentrations. Apoptotic cells labelled with activated caspase 3/7 show bright green nuclei. Each image side by side correspond to different representative pictures taken of same treatment.

The information obtained in this assay is consistent with the cell counting, where a slight decrease in cell number in OA2 treatment was observed, although not statistically significant. LA2 did not show less evidence of apoptosis than the other treatments, but according to the assay of viability and cell counting this treatment stimulates growth. So this is probably due to stimulation of proliferation.

4. Effect of FAs on total lipid content

The total amount of lipid in each of the samples and the total cell protein content, were determined. The results are shown in Table 1.

Table 1. Results of quantification of PL (ug) and protein, P (mg) per 900 uL of sample. R1, R2, R3 are the three independent experiments; each measurement was carried out in duplicate. Legend: Control – Without FAs; OA1- oleic acid (C18:1n9 1 ug/mL); LA1- linoleic acid (C18:2 n-6 1 ug/mL); LA2- linoleic acid (C18:2 n-6 2 ug/mL);

PL (ug)								
	Control		OA1		LA1		LA2	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
R1	1212,90	107,30	1172,00	59,59	1032,00	9,53	1385,90	129,24
R2	242,70	2,13	282,50	4,00	565,90	9,33	450,80	19,19
R3	1701,02	79,39	2176,86	61,81	2066,85	128,94	1988,11	3,73
Reptetition of quantification								
	Control		OA1		LA1		LA2	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
R1	536,31	3,17	487,64	6,79	477,07	39,85	710,81	92,37
R2	214,52	1,20	276,00	24,45	639,73	38,94	393,19	21,73
R3	1395,04	81,96	1743,72	28,07	1733,48	55,24	1590,36	123,23
P (mg)								
	Control		OA1		LA1		LA2	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
R1	8,48	0,66	10,38	0,00	9,37	2,53	9,35	0,24
R2	6,74	0,03	7,87	0,21	7,14	0,40	5,95	0,09
R3	10,19	0,56	9,77	0,95	10,31	0,11	9,66	0,19

A large difference in the protein concentration was observed, and this is directly related to the cell number. Still, PL quantification also revealed differences between independent experiments. So, to make sure that the variations of total PL levels were due to treatment with FAs, to which the cells were exposed, and not to differences in the number of cells

of each independent experiment, the values of quantification were normalized to total cell protein content, the results are presented in Figure 16.

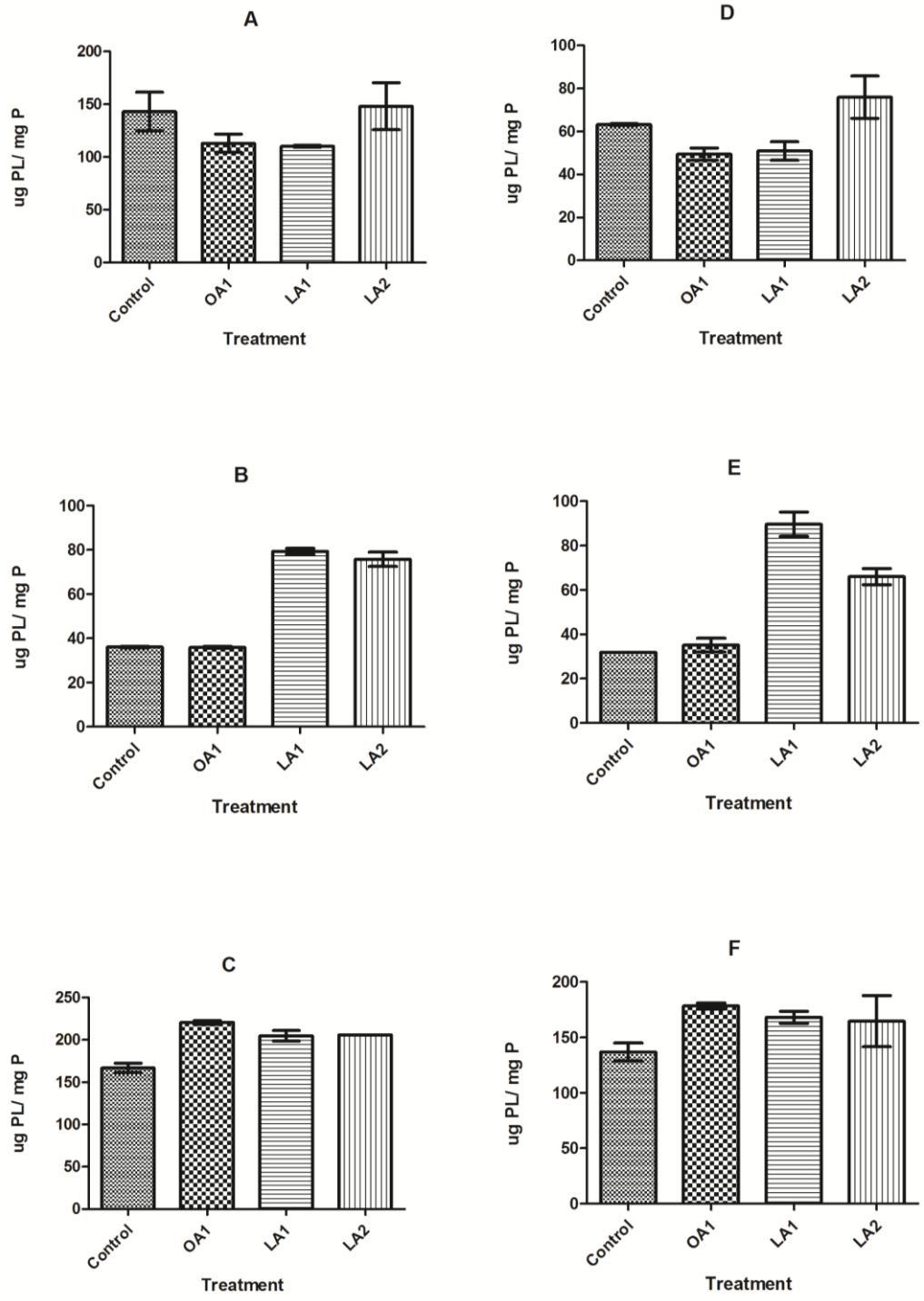

Figure 16. Amount of lipid in each sample expressed by ug of PL per mg of protein. A, B, and C correspond to the first quantification of R1, R2 and R3; respectively; D, E and F correspond to the second quantification. Mean +/- SD from duplicates is shown. Note: *the difference in the PL amount between A and D, which correspond to R1is due to the fact that in D, some sample had already been used for other purposes.*

It is possible to see that the two quantifications gave similar results, showing that the difference observed between each independent experiment is not due to experimental error, but to variability between samples. To better visualize possible differences in total PL amount, the values were normalized relative to control (Figure 17).

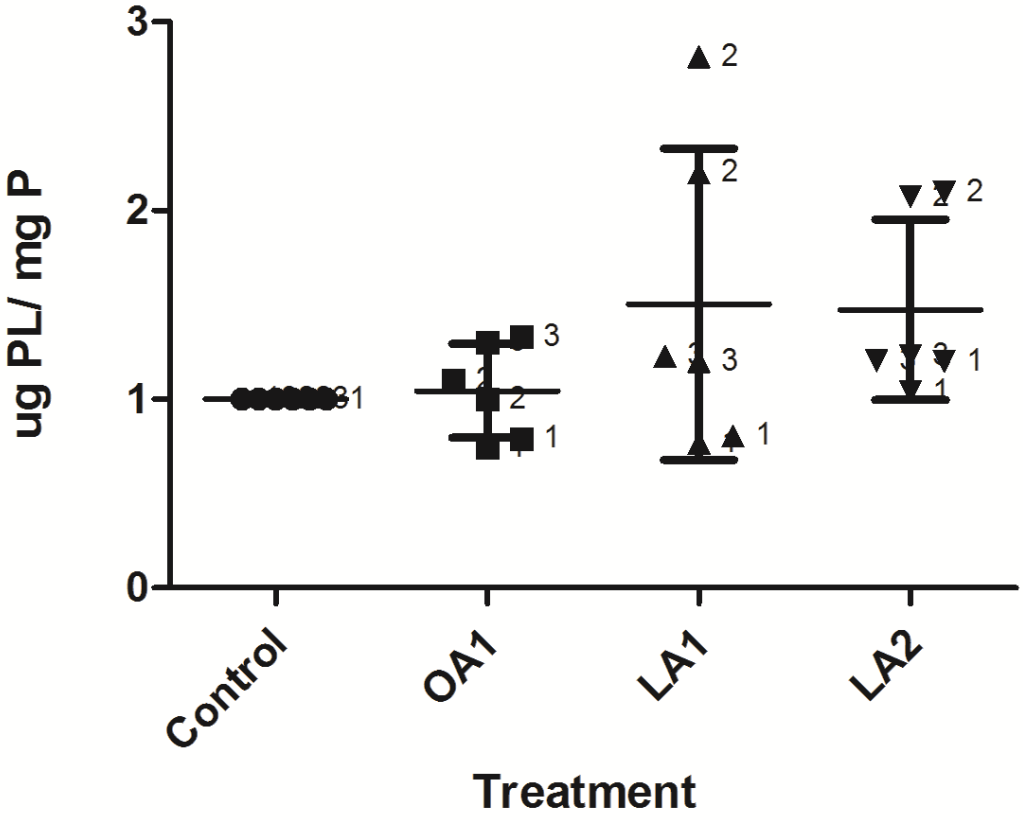

Figure 17. Graphic representation of the amount of PL in each sample expressed by ug PL / mg protein relative to control (mean +/- SD). 1, 2 and 3 correspond to values from R1, R2 and R3 of the two quantifications. Statistical differences were analyzed with One-way ANOVA and Dunnett's post-hoc test, but no differences were found.

Taking the results into account, differences in total PL content were not considered significantly different between the treatments.

5. Effect of dietary fatty acids on cellular fatty acid levels

In order to see if exogenous FAs affect cellular FA levels, analysis of modification of FA profile (free and esterified) of the total lipid extract from T-47D cells supplemented with different FA treatment (Figure 18) was performed by using GC-MS methodology.

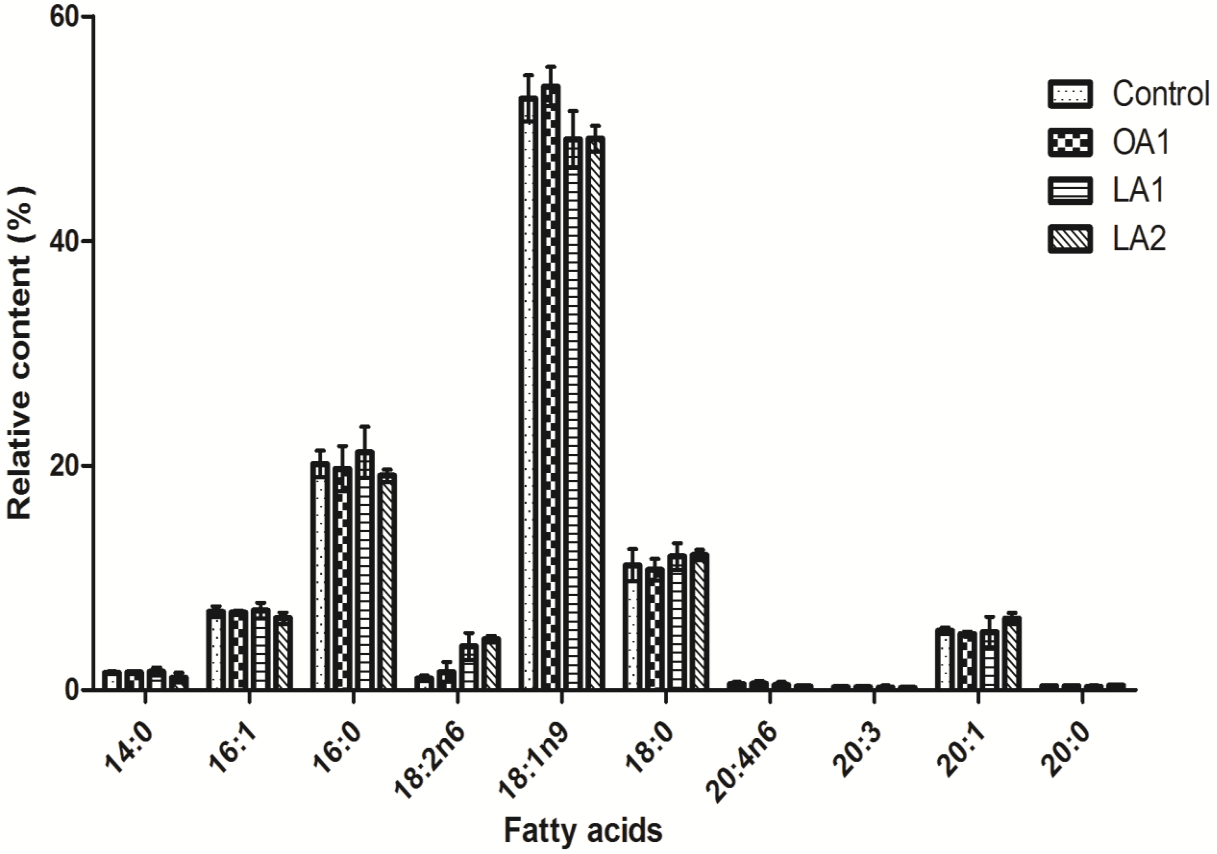

Figure 18. GC-MS quantification of FAs in total lipid extract. The results are relative to total FAs and are presented as Mean +/- SD.

Observing the results we can see that there are some alterations in the FA profile. The major difference is related to 18:2 n-6 in cells treated with LA1 ($p<0.05$) and LA2 ($p< 0.01$), that showed an increase in the relative content (Figure19). This suggests that 18:2 n-6 from the medium is being accumulated directly in lipids of cells but probably not metabolized into other FAs, namely 20:3 or 20:4n-6.

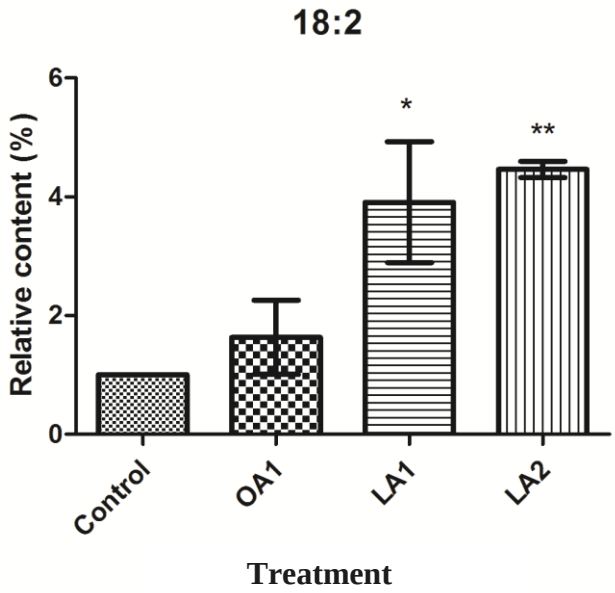

Figure 19. **The relative content of 18:2 FA in T-47D cells.** The results are presented as FA Mean +/- SD normalized relative to untreated control from three independent experiments. Statistical significance was measured with One-way ANOVA and Dunnett's post-hoc test,*: $p<0.05$; **: $p< 0.01$

To better analyze the FAs present in minor quantities, the results were normalized to the untreated control and plotted with a smaller scale. However, differences were not statistically significant (Figure 20).

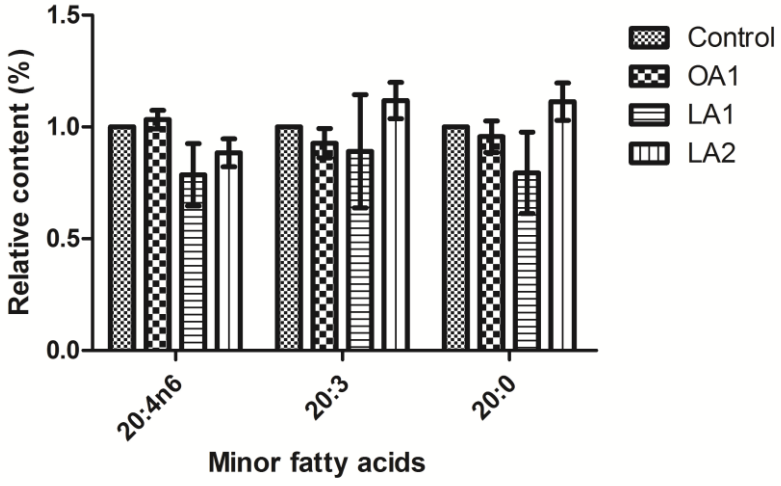

Figure 20. GC-MS quantification of minor FAs in total lipid extract from T-47D cell line. Mean +/- SD normalized relative to untreated control from three independent experiments. Statistical significance was measured with One-way ANOVA and Dunnett's post-hoc test, but no differences was found.

LA can change the cell FAs composition, and may be stored as PLs. So if the accumulation of 18:2 n-6 occurs in the PLs, which are the major structural components of biological membranes, it will alter the ratio between saturated and unsaturated FAs. This effect will change the lipid composition of membrane, which may lead to variance in membrane fluidity, possibly leading to alterations on proteins of membrane like the conformation of certain transporters, receptors, and enzymes that are sensitive to changes in the structure of their lipid microenvironment, leading to changes in protein activity (84,85).Broadly, alterations in membrane fatty content change the membrane-receptor and cell-to-cell communications resulting in abnormal and unregulated cell function (85).

FA also could be incorporated into storage lipids (TG and CHL esters), or directly oxidized to produce energy (86). Besides that, FA may also influence the function of the cell as free FA (FFA). In literature has been proposed the different mechanisms by which FFA can influence progression of cancer. For example, they can interact directly with enzymes or receptors and depending on their structure they can modulate their functions differently. Furthermore, they can acylate proteins and, which is important for anchoring certain proteins in membranes or for folding of the proteins, and it is crucial for the function of these proteins (86). For example, protein modification with SFA can promote their association with CHL of lipid rafts, whereas PUFA, like 18:2 n-6 exclude proteins from these structures leading to changes on the activity of signaling complexes that are associated with membrane rafts. Still, FFA and their derivatives (eicosanoids or acyl-CoA) may interact with nuclear receptor proteins that bind to certain regulatory regions of DNA altering the transcription of genes (29,87).In a study with T-47D cells, the treatment with LA provided information that LA may influence cell cycle progression and global gene expression. They have found that treatment of T-47D cells with LA is associated with an up-regulation of oncogenes, modulation of growth factor signaling and regulation of the cell cycle machinery. These include genes involved in transcriptional regulation, cell cycle control, G protein and MAP kinase signaling pathways, supporting the data that linoleic acid increases T-47D cell proliferation (84).

Therefore, the increase of cellular 18:2 n-6 in cells treated with LA1 and LA2 could be incorporated in PLs or to storage lipids (TG and CHL esters). Also the FFAs could be captured by cells and then activated to produce energy, to modulate enzymes or produce eicosanoids, or even to bind to transcription factors that regulate gene expression.

6. Total triglycerides quantification

FAs can be used to generate many different types of lipids, for example they can be converted into TGs via the glycerol phosphate pathway (29). Therefore it is important to understand if the FAs supplemented in the medium could be stored as TG.

The analysis of TGs was carried out using a colorimetric enzymatic method (Figure 21). As we can see, the results were not reproducible, even in the control samples. These assays were originally devised to measure TG levels in human serum samples which travel in plasma in soluble lipoprotein complexes. Thus, the lipase used in the colorimetric assay kits can efficiently form free glycerol (the measured product) in these conditions. However, our sample is a total lipid extract (hence, it is very hydrophobic), so what could be happening is that the approach used for solubilization is not effective and the liposomes used to mimetize the lipoprotein, are not correctly formed. Moreover, we tried to optimize the method of quantifying the TG, by adding 5% Triton X100 to the ammonium hydrogen carbonate buffer to try to solubilize better the samples. Triton X100 has been used in other procedures with little evidence of loss of lipase enzyme activity (88).

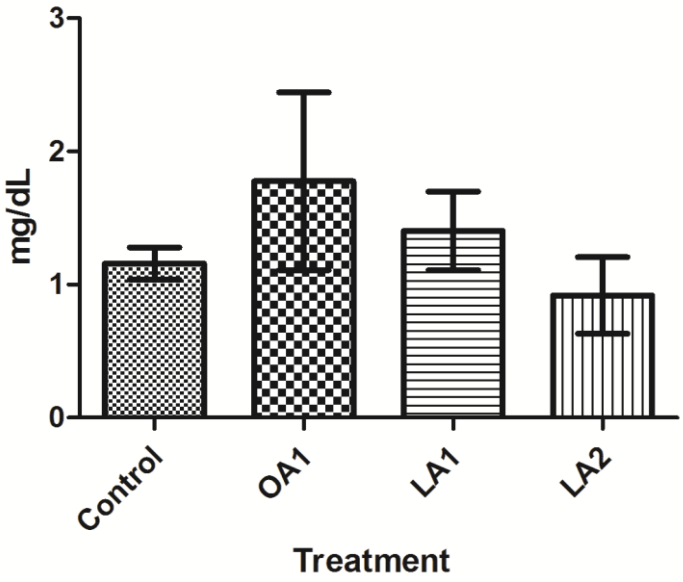

Figure 21. Quantification of TG in total lipid extract from cells treated with different FAs. TG values (mg/dL) were normalized to protein content and related to the untreated control**.** Mean +/- SD from three independent experiments **t**-test was used to analyze significant changes.

A pilot study, was carried out in an attempt to predict an appropriate sample dilution and to analyze if addition of 5% Triton X100 would improve the reproducibility of the assay (Figure 22). Since 5% of Triton X100 and greater dilution improved the assay, we proceeded to the measurement of TG in our samples (Figure 23).

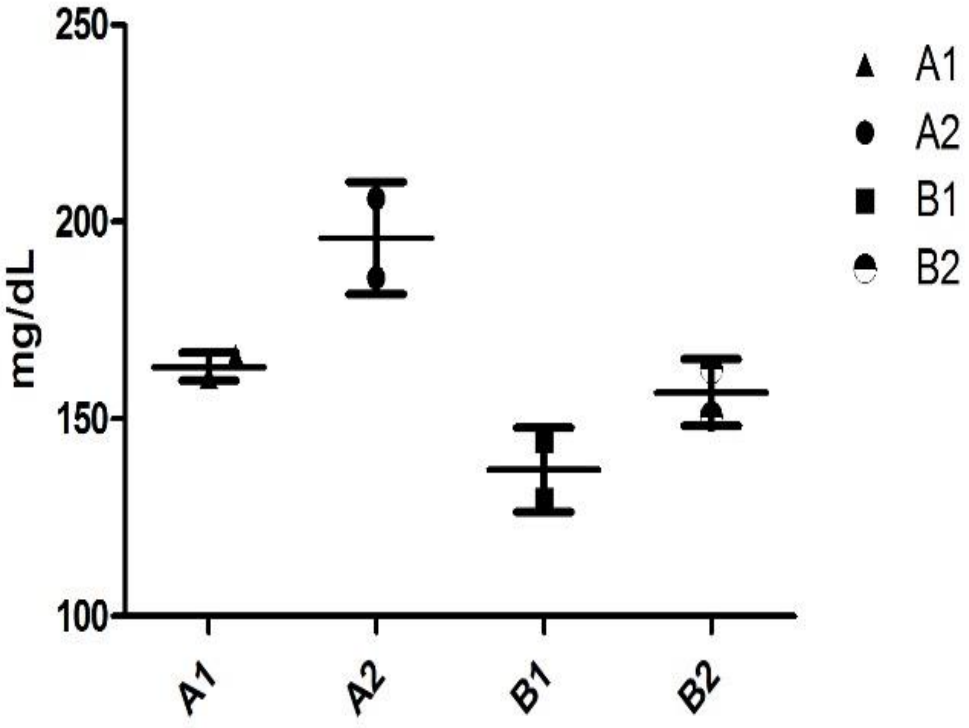

Figure 22. Pilot test to see which condition better solubilized the sample. The samples had a known concentration of TG (220 mg/dL). Legend: A1 – Ammonium hydrogen carbonate buffer 5 mM pH 7.4 dilution than 1/5; A2 – Ammonium hydrogen carbonate buffer 5 mM pH 7.4 with 5% Triton X100 at a dilution 1/5; B1 - Ammonia hydrogen carbonate buffer 5 mM pH 7.4 at a dilution 1/3; B2 – Ammonium hydrogen carbonate buffer 5 mM pH 7.4 with 5% Triton X100 at a dilution 1/3. Mean +/- SD is shown.

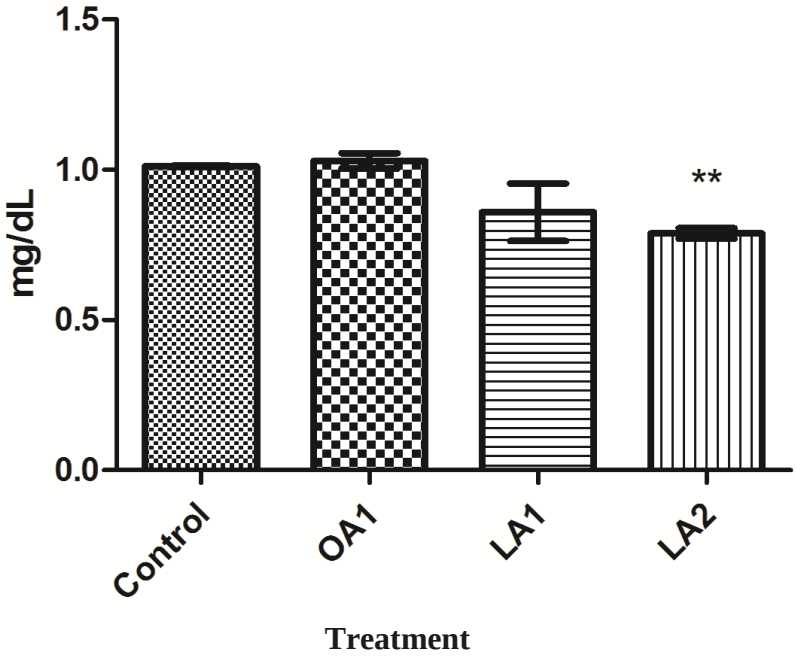

Figure 23. **Quantification of TGs in total lipid extract from cells treated with different FAs.** TG values (mg/dL) were normalized to protein content and related to the untreated control. Mean +/- SD from three independent experiments **t**-test was used to analyze significant changes. **: $p<0.01$.

The results indicate that total TGs were decreased in cells treated with LA2 compared with other conditions. Increased cancer cell proliferation requires the rapid synthesis of lipids for the generation of biological membranes (86,89). Since the culture medium used is a medium that only has essential nutrients for growth, was hypothesize that cells treated with LA2 that have increased metabolic activity and cellular proliferation might be using TGs to provide metabolic substrates for energy production (via FA oxidation) and membrane lipids, decreasing the levels of TGs total content. Also, TGs might be used to produce metabolites important for cell growth and proliferation (86). Thus, even though LA2 results in increased cellular 18:2 n-6 levels, it is possible that it does not accumulate as part of the TGs.

7. Total cholesterol quantification

CHL is essential for the normal function of all animal cells. It is an important membrane component that modulates the fluidity of the lipid bilayer, especially in lipid rafts. CHL alterations affect lipid raft structure and also might alter the receptors function, so the levels of CHL is a key factor to determine lipid raft stability and organization (38).

The analysis of CHL was carried out using a colorimetric enzymatic method. In this assay the CHL esters are hydrolyzed by CHL esterase to form free CHL, which, after oxidation with CHL oxidase form hydrogen peroxide. The peroxide reacts with phenol, 4-aminoantipyrine via oxidation catalyzed by peroxidase, yielding a red-colored substance (quinonimine) that allow measure the CHL by absorbance. But just like in TG quantification, when colorimetric assays are used to evaluate suspensions of insoluble fat, the solubilization must be effective for the cleavage to occur completely and yield an accurate measure. We observed that when analyzing total lipid extracts, the results were not reproducible, even in the control sample (Figure 24).

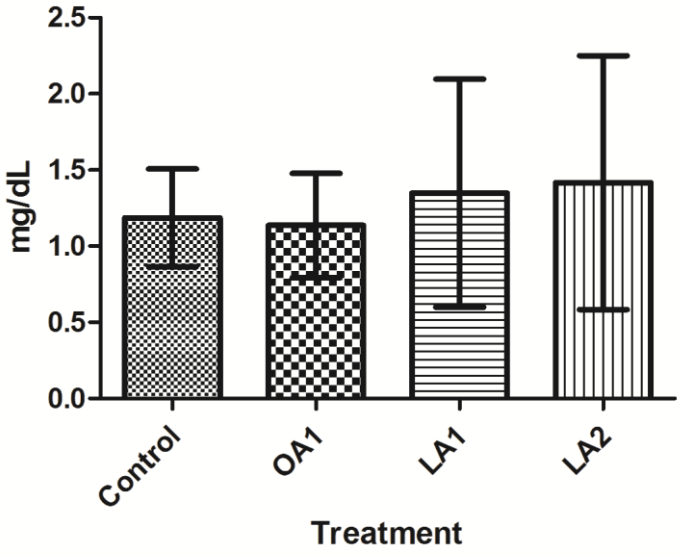

Figure 24. Quantification of CHL in total lipid extract from cells treated with different FAs. CHL values (mg/dL) were normalized to protein content and related to the untreated control. Mean +/- SD from three independent experiments **t**-test was used to analyze significant changes.

So, a pilot test was conducted with the same conditions as the TG pilot test (Figure 25). The condition more effective was the buffer mixed with 5% of Triton X100 with a dilution of 1/3. However, we could not accurately measure the concentration present in the sample and the measurement of concentration of CHL was repeated (Figure 26).

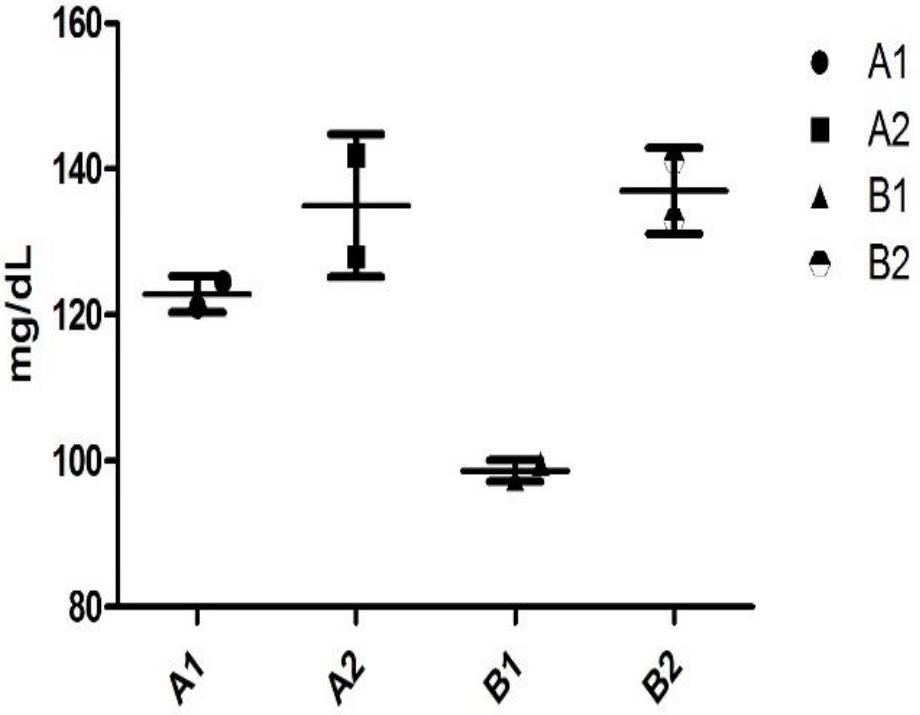

Figure 25. Pilot test to see which condition better solubilized the sample. The samples had a known concentration of CHL (200 mg / dL). Legend: A1 - Ammonium hydrogen carbonate buffer 5 mM pH 7.4 dilution than 1/5; A2 - Ammonium hydrogen carbonate buffer 5 mM pH 7.4 with 5% Triton X100 at a dilution 1/5; B1 - Ammonia hydrogen carbonate buffer 5 mM pH 7.4 at a dilution 1/3; B2 - Ammonium hydrogen carbonate buffer 5 mM pH 7.4 with 5% Triton X100 at a dilution 1/3. Mean +/- SD is shown.

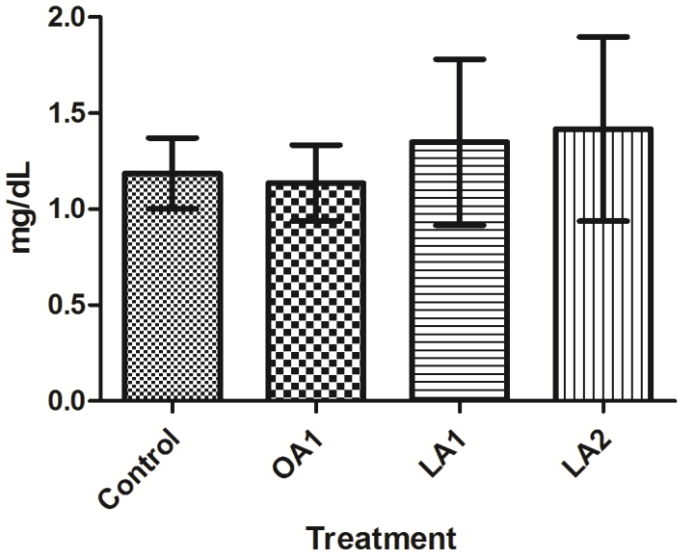

Figure 26. Quantification of CHL in total lipid extract from cells treated with different FAs. CHL values (mg/dL) were normalized to protein content and related to the untreated control. Mean +/- SD from three independent experiments **t**-test was used to analyze significant changes.

The results were not reproducible and none showed significant differences when compared to other groups so curiously, this approach worked to the TG but not for CHL. Therefore, another approach should be implemented to measure the total CHL. It could be measured by a multistep process in which the lipids are first extracted with organic solvents, like we did in our stud, in order to have a total lipid extract. This step would be followed by chromatographic methods such as TLC to separate the CHL and then the quantitative analysis of the samples. This is more laborious and requires the use of strong acids and other dangerous chemicals so the use of enzymatic reagents for tissue lipid analysis was clearly more desirable and faster however in the case of CHL, it is not the most recommended.

8. Effect of fatty acids on the relative levels of phospholipid classes

Many cellular functions and responses are affected when the membrane lipid composition is modified because membrane fluidity and structure changes. These alterations may affect the conformation of certain transporters, receptors, and enzymes

that are sensitive to changes in the structure of their lipid microenvironment, leading to changes in protein activity (85).

In order to see if exogenous FAs affect PL of membranes, quantification of each PL class were performed. Each PL class was separated from the total lipid extract by TLC a very valuable technique, which is fast and simple (90). Identification of PL classes was carried out by comparison with known standards also applied in the TLC plate. The classes identified were LPC, SM, PC, PI, PS, PE and CL (Figure 27).

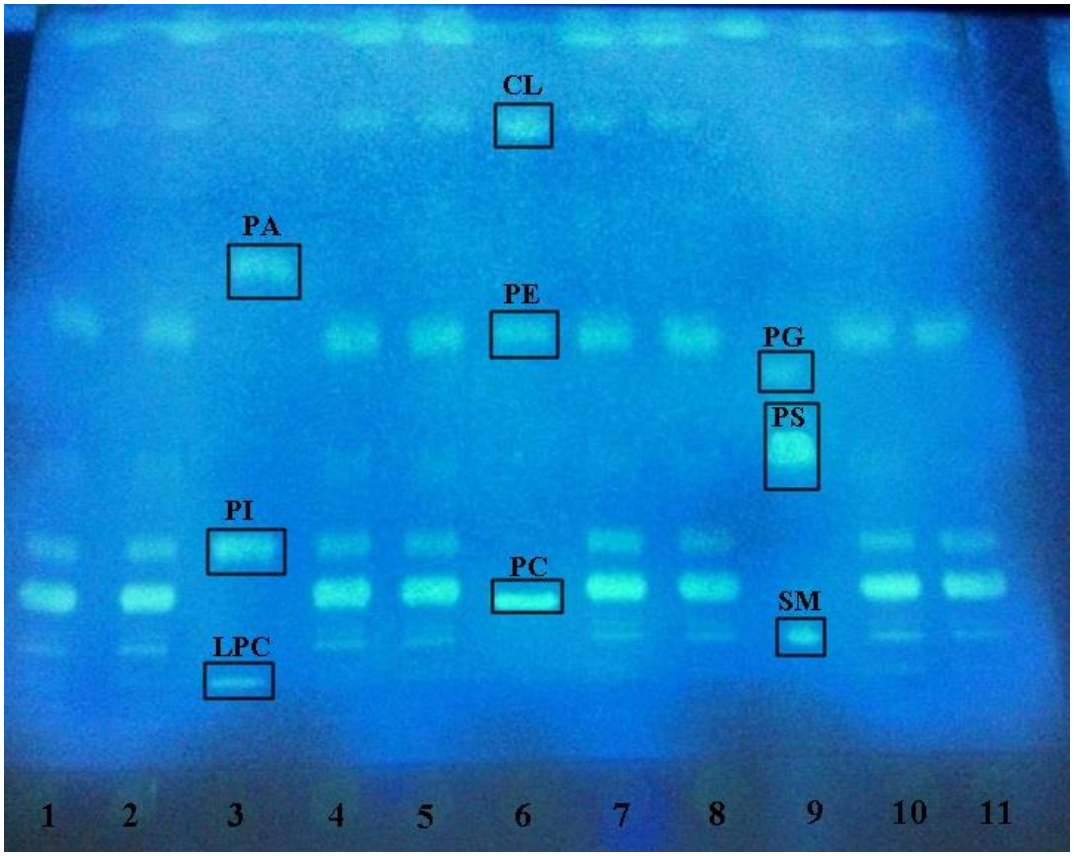

Figure 27. Representative TLC separation of 30 ug total lipid extract obtained from T-47D cell line treated with different FAs. Lane 1-2 – Control extract; Lane 3- PA, PI and LPC standards; Lane 4-5 – OA1 extract; Lane 6 – CL, PE and PC standards; Lane 7-8 - LA1 extract; Lane 9 – PG, PS and SM standards; Lane 10-11 – LA 2 extract. Abbreviations: PC - Phosphatidylcholine; PS - Phosphatidylserine; PE - Phosphatidylethanolamine; SM - Sphingomyelin; PG - Phosphatidylglycerol; PI - Phosphatidylinositol; PA - Phosphatidic Acid; CL - Cardiolipin; LPC – Lysophosphatidylcholine; OA1 – Oleic acid 1 ug/mL (18:1 n-9) ; LA1 – Linoleic acid 1ug/mL (18:2 n-6); LA2 - Linoleic acid 2 ug/mL.

After the separation of each PL class, the spots were scratched and the amount of phosphorous in each spot was quantified. Thereafter the % of each class was calculate relative to total PL. The results obtained are shown in Table 2.

Table 2 Percent (%) of each class of PL in three independent experiments (R1, R2 and R3). Values in bold were not included in the graph.

	% retative content of PL									
	Control					OA1				
Classes	R1		R2	R3		R1		R2	R3	
LPC	**1,4013**	4,7375	6,2720	4,7421	4,0431	2,3437	3,6400	**7,1049**	3,6531	3,0367
SM	7,8655	6,3605	6,4408	8,6244	6,9872	6,6332	9,0768	7,6400	8,6751	8,1178
PC	32,9536	36,7529	30,7696	30,0278	34,6125	36,8363	38,8816	29,8168	28,9044	34,3462
PI	12,8831	11,3473	**14,1754**	11,8596	11,3775	13,6201	9,0486	11,2668	11,8391	11,2998
PS	10,9032	13,1276	13,4722	10,8823	12,3986	**10,2751**	13,8183	16,9745	14,1792	15,1195
PE	17,1775	18,0768	15,7223	**21,9872**	17,9635	17,0694	17,5829	16,3502	**20,9012**	18,3544
CL	16,8158	9,5973	13,1477	11,8766	12,6176	13,2222	7,9519	10,8467	11,8480	9,7257

	% retative content of PL									
	Control					LA1				
Classes	R1		R2	R3		R1		R2	R3	
LPC	**1,4013**	4,7375	6,2720	4,7421	4,0431	4,4132	5,8304	5,9136	5,4703	**2,5639**
SM	7,8655	6,3605	6,4408	8,6244	6,9872	8,6379	6,5626	7,4467	7,0772	6,4228
PC	32,9536	36,7529	30,7696	30,0278	34,6125	40,6621	36,2977	30,0059	28,4222	32,9590
PI	12,8831	11,3473	**14,1754**	11,8596	11,3775	8,8642	9,6843	12,3747	13,6022	12,7279
PS	10,9032	13,1276	13,4722	10,8823	12,3986	**8,4915**	13,3809	15,6600	11,8621	13,3726
PE	17,1775	18,0768	15,7223	**21,9872**	17,9635	**15,6915**	20,4667	19,3833	22,9658	21,5640
CL	16,8158	9,5973	13,1477	11,8766	12,6176	13,2397	7,7775	9,2158	10,6002	10,3898

	% retative content of PL									
	Control					LA2				
Classes	R1		R2	R3		R1		R2	R3	
LPC	**1,4013**	4,7375	6,2720	4,7421	4,0431	4,9458	3,6278	5,3407	4,2743	3,1355
SM	7,8655	6,3605	6,4408	8,6244	6,9872	8,6551	6,7703	7,8430	7,5810	7,3602
PC	32,9536	36,7529	30,7696	30,0278	34,6125	37,3758	39,1786	29,8781	29,2822	32,4699
PI	12,8831	11,3473	**14,1754**	11,8596	11,3775	10,7747	10,5092	**14,4162**	9,9826	10,0760
PS	10,9032	13,1276	13,4722	10,8823	12,3986	**7,8676**	12,8887	17,1799	18,0112	13,7899
PE	17,1775	18,0768	15,7223	**21,9872**	17,9635	18,7939	19,0772	16,2462	17,9936	**21,2499**
CL	16,8158	9,5973	13,1477	11,8766	12,6176	11,5872	7,9482	9,0958	12,8751	11,9187

Values in bold represent values excluded to perform the graph because there are very different from other values in the same sample and are most probably related to errors in the methodology. The results are represented in Figure 28.

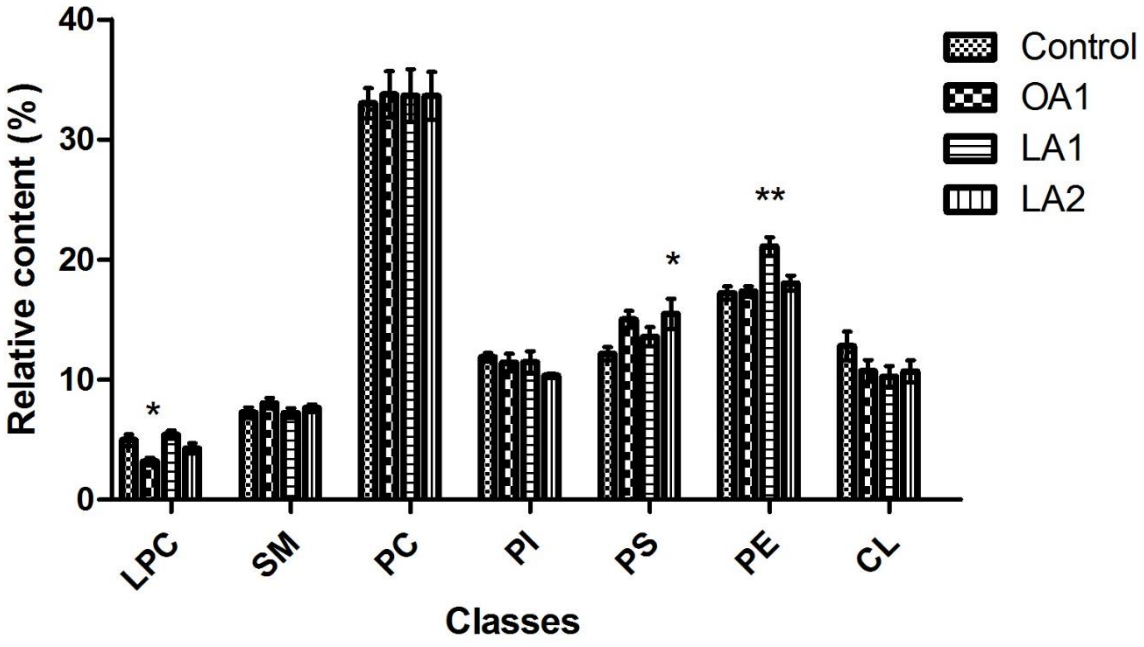

Figure 28. Relative PL content (%) in each class of T-47D cell line treated with different FAs. PL classes were separated by TLC and phosphorous content in each spot was related to total phosphorous content in the sample. The results are presented as mean +/- SEM from three independent experiments is shown. Statistical significance was measured with One-way ANOVA and Dunnett's post-hoc test *: $p<0.05$, **: $p<0.01$.

The most abundant PL class in all treatments was PC, followed by PE. Cells treated with LA1 showed an increase in the level of PE, while cells treated with LA2 showed an increase in PS. A decrease in LPC in cells treated with OA was observed. Finally, in CL it was observed a tendency to decrease in all treatments, especially in LA1, and PI was also decreased in LA2. However, these differences were not statistically significant. So, although there are slight changes in PL classes only LPC, PS and PE were significantly changed by OA, LA2 and LA1, respectively. A study of Doria *et al* (2012) that compared the profile of PLs from mice mammary epithelial and breast cancer cell lines, showed that decreased levels of LPC are associated to less malignancy, which is in agreement with the results presented. PS levels didn't showed differences (67). Another study of Doria *et al* (2013), which compared the profile of PLs from human mammary epithelial and breast cancer cell lines, showed that relative content of LPC where similar in all cell lines. Also, the PS relative amounts showed an increase in malignant cells but it wasn't statistically

significant. Curiously, in both studies PE was found increased in non-malignant cells when compared with malignant cells (91).

Based on the PL class biosynthetic pathways, we can speculate on possible enzymes regulated by the FA treatment. In Figure 29 the main pathways of PL metabolism are presented.

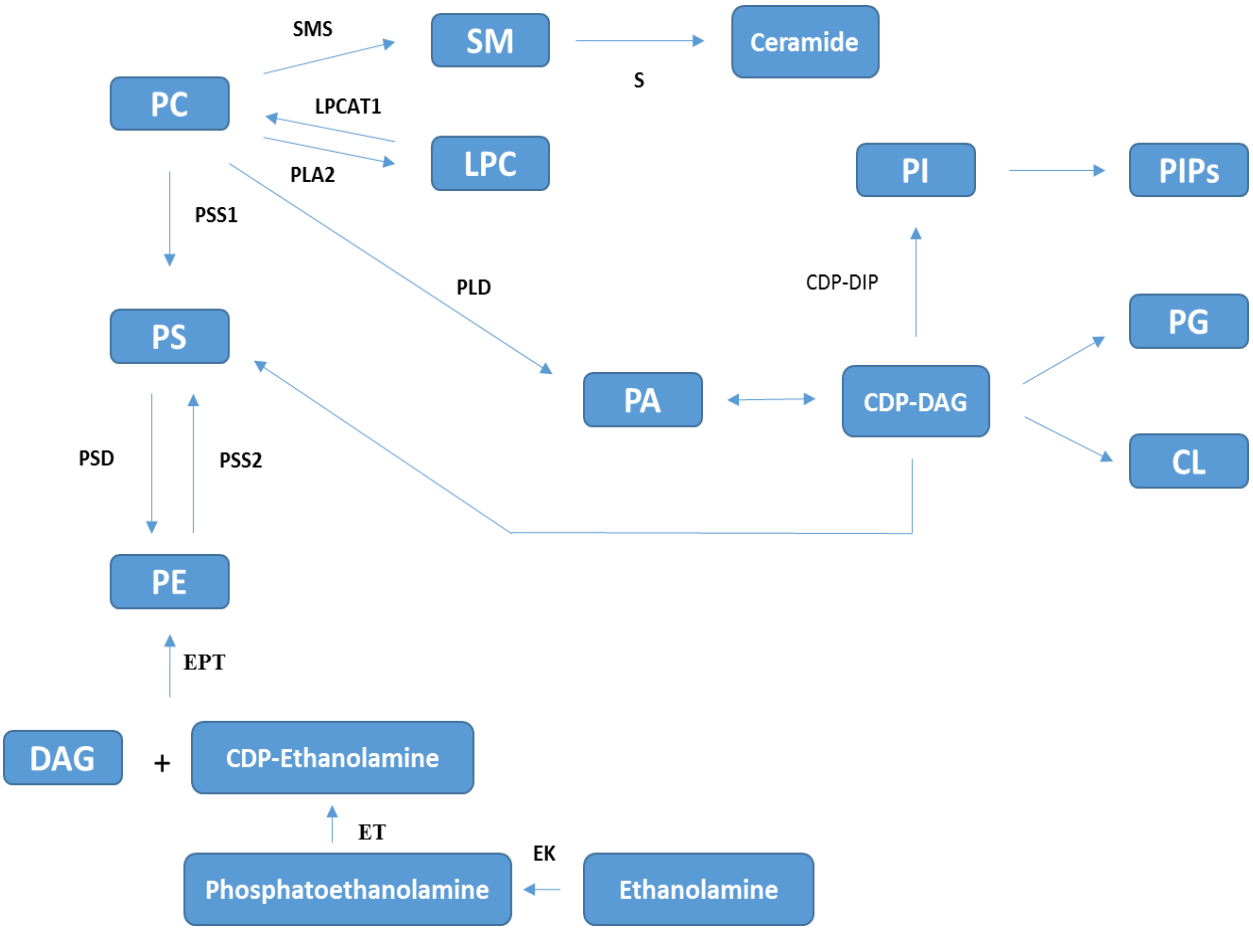

Figure 29. Main pathways of biosynthesis of PLs. PA: Phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PI: phosphatidylinositol, PS: phosphatidylserine, LPC: lysophosphatidylcholine, CL: cardiolipin, SM: sphingomyelin, CDP: cytidine diphosphate, DAG: diacylglycerol,PIP: phosphatidylinositol phosphate. PLD: phospholipase D, PLA2: phospholipase A2, LPCAT1: lysophosphatidylcholine acyltrasferase1, PSD: phosphatidylserinedescarboxilase, PSS1: phosphatidilserine synthase 1, PSS2: phosphatidilserine synthase 2, S: sphyngomielinase, SMS: sphyngomilelin synthase, CDP-DIP: CDP-DAG inositol phosphatidyltransferase, EPT: CDP–ethanolamine: DAG ethanolamine phosphotransferase, ET: CTP:phosphoethanolamine cytidylyltransferase, EK: ethanolamine kinase. Note: *this scheme is a compilation of information based on literature referred on this section.*

PE plays a major role in membrane curvature and this influences structure, folding, and activity of membrane proteins. Synthesis of PE can occur by two pathways. The first requires using the ethanolamine as the substrate. Ethanolamine is activated by phosphorylation to form the headgroup of PE, the phosphoethanolamine by ethanolamine kinase (EK) and then phosphoethanolamine reacts with CTP and couple with cytidine-diphosphate (CDP) through the action of CTP: phosphoethanolamine cytidylyltransferase (ET). In the final step, phosphoethanolamine from CDP-phosphoethanolamine is attached to the *sn*-3 position of DAG to yield PE by CDP-ethanolamine: DAG ethanolamine phosphotransferase (EPT) (92). The second pathway involves the decarboxylation of PS by the PS decarboxylase (Figure 29). In the case of LA1 treatment, the increase verified in PE class could be caused by the increase of ethanolamine phosphorylation (by EK) (93). These reasons are more probable than PS descarboxilation because the levels of PS did not decrease, in fact it could be seen a slight increase of PS in LA1 treatment.

PS is a PL that is present in the inner leaflet of the cell membrane and have other important functions like an essential cofactor that binds to activate the protein kinase C, that is a key enzyme involved in the signal transduction. PS is increased with LA2 treatment that may be associated with PE metabolism, by action of PSS2. So expression of this enzyme may be induced in T-47D cells treated with LA2.

LPC can be derived from PC (Figure 29) due to the action of phospholipase A2. It has been proposed that LPC can stimulate cell signaling and cell migration through its action as an extracellular ligand to specific receptors (coupled to G proteins). Further signaling effect may activates the specific phospholipase C, which will releases DAG and inositol triphosphate with resultant increases in intracellular Ca^{2+} and activation of protein kinase C or by the action of autotaxin (ATX). LPC can be converted to LPA, a potent signaling molecule, by the action ATX via it lysophospholipase D activity. Both LPC as the LPA can lead to a stimulation of cell proliferation and migration (94). For example, ATX is found in extracellular fluid surrounding the tumors , this enzyme is associated to invasiveness and higher plasma LPA and LPC levels on patients with ovarian and breast cancer, compared with healthy controls (95,96). Therefore, it was hypothesize that the decrease of the total content of this class in cells treated with OA could reduce the action of these PL, leading to a decrease of LPA and consequently lower stimulation of proliferation.

In summary, the PS and PE are altered in a medium supplemented with LA altering the structure of the membrane. The conformation or quaternary structures of certain

transporters, receptors and enzymes located in membranes are sensitive to changes in the structure of their lipid microenvironment, and that might affect a number of cellular functions, that could lead to cell growth and proliferation. We speculate that 18:2 n-6 increased in the LA1 and LA2 treatment may be esterified PS and PE in these samples, which in turn can be related to the increased metabolic activity/cell number. The decrease of TG can be due to beta-oxidation to provide energy for cell growth and metabolism. LPC is altered in OA medium, which could be related to a decrease of stimulation of cell proliferation in breast cancer cells, through the decrease of production of metabolites that enhance the cell proliferation like LPA.

IV. Conclusion

IV. Conclusion

The aim of this work was to evaluate if the lipidome of the T47-D breast cancer cell line may be modified by different dietary FAs supplementation and to try to evaluate if these changes could affect viability, growth and apoptosis.

The lipidomic methods used showed to be valuable for analysis of fatty acids effects on cancer cells. With this approach, it was possible to identify differences in the lipidome of cancer cells treated with OA or LA when compared to control. An increase of relative amounts of PE and PS were found in cells treated with LA and the increase of the FA 18:2 in cells was also observed. Furthermore, a decrease of LPC was found in cells treated with OA. Also total TGs were decreased in cells treated with LA2. Therefore it was possible to conclude that supplementation with LA is associated with significant changes in lipidome that may be related to the effects on cell survival and integrity, and that OA may have the opposite effect, due to an increase of apoptosis.

Finally, this work contributes to a better understanding of the influence of dietary FAs on the lipidome of T-47D cells and this alterations are involved on changes in cell number and apoptosis, being LA associated to a stimulation of growth of this cells and OA associated with inhibition of cell growth. These findings may lead to new perspectives in the identification of specific mechanisms that may be helpful to understand the process behind this disease. Nevertheless, more studies are necessary to take more conclusions about how FAs promote the effects observed.

V. Future Perspectives

V. Future perspectives

In the future, this work could be completed by a more detailed analysis of the lipidome by using LC-MS. This approach will allow to identify the modifications at the molecular level, as well as if the dietary FA supplemented are being incorporated in any specific class of PLs. Thus, identification of PL classes and the changes in their fatty acyl chains will open new possibilities to exploration of these alterations in cancer. From these relation new biomarkers might arise to prevent cancer and a basis to research and develop new therapeutic opportunities.

VI. References

VI. References

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